Neutrophil activation in health and connective tissue diseases

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Award date:
1987

Awarding institution:
University of Bath

NEUTROPHIL ACTIVATION IN HEALTH AND CONNECTIVE TISSUE DISEASES

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for the degree of PhD

of the University of Bath

1987

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This thesis is dedicated to my family, for their love and support, without which I would never have got this far.

To PP

Is it the time, flying along,

That makes me feel something's wrong,

You know me well, the tale I tell,

Is only halfway there 'cause you,

You made me smile when I was down,

You, you made me happy being around,

So I'll always sing my song for you.

Acknowledgements

I would like to thank my supervisor, Dr Nick Hall for his patience and advice over the past three years (I hope that the experience will not lead to any long lasting psychological problems).

I am also indebted to the very special people at the Bath Arthritis Research Centre who gave their blood so freely and made the whole experience a happy and memorable one.

Finally I thank Celia for her ultra-rapid typing (and for the bruises)^2

Abbreviations

AA	arachidonic acid
AC	adenylate cyclase
ADP	adenosine diphosphate
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
B_{245}	cytochrome B_{245}
pBPB	parabromophenacyl-bromide
C3/C5	complement components
CM	calmodulin
CGD	chronic granulomatous disease
DAG	diacylglycerol
DMSO	dimethylsulphoxide
DPI	diphenylene iodonium
DNA	deoxyribonucleic acid
FVIII Rag	factor VIII related antigen
FAD	flavin adenine dinucleotide
FMLP	n formyl 1 methionyl 1 leucyl 1 phenylalanine
G-SH	glutathione
HMPS	hexosemonophosphate shunt
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroxygperoxyeicosatetraenoic acid
HRPO	horseradish peroxidase
HX	hypoxanthine
Ig	immunoglobulin
IP_3	inositol 145 triphosphate
LT	leukotriene

MCTD	mixed connective tissue disease
NADPH	reduced nicotinamide adenine dinucleotide
OZ	opsonized zymosan
PBS	phosphate buffered saline
PC	phosphatidylocholine
PE	phosphatidylethanolamine
PG	prostaglandin
PI	phosphatidyinositol
PKA	cAMP dependent protein kinase
PKC	protein kinase C
PMA	phorbol myristate acetate
PR	primary Raynauds
SD	standard deviation
SLE	systemic lupus erythematosus
SS	systemic sclerosis
TMB-8	8 (N, N diethylamino)-octyl 3, 4, 5 trimethoxy-benzoate hydrochloride
XDH	xanthine dehydrogenase
XO	xanthine oxidase

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Summary

Neutrophil activation following exposure to particulate matter or inflammatory mediators is accompanied by a marked increase in oxidative metabolism and the generation of superoxide and hydrogen peroxide. This thesis has looked at the oxidative response of neutrophils exposed to FMLP (a synthetic bacterial peptide) aggregated IgG (to represent immune complexes) and PMA (an experimental ligand which bipasses the cell surface). The inhibitory effects of diphenylene iodonium confirmed that all basal and stimulated H_2O_2 was derived from NADPH-oxidase. The molecular mechanisms of NADPH oxidase activation were investigated using two groups of pharmacological agents. Inhibitors of the phospholipase A_2 enzyme reduced basal and FMLP stimulated H_2O_2 production but were relatively ineffective against H_2O_2 stimulated by PMA and aggregated IgG. Inhibitors of PLA_2 however, were ineffective at inhibiting the neutrophil chemotactic response to FMLP. Thus, it appears that the molecular mechanisms of neutrophil activation vary not only with the stimulus employed but also with the nature of the ultimate response.

Agents which activate adenylate cyclase and raise intracellular cAMP were also found to inhibit neutrophil H_2O_2 production. Their effects were dependent upon a synergism between the small cAMP response produced during neutrophil stimulation and that produced by the classical stimulants of adenylate cyclase - the overall result being to convert an apparently innocuous rise in cAMP to one that inhibits neutrophil activity.

Finally, the oxidative metabolism of neutrophils from patients with connective tissue disease were compared with healthy control subjects. Elevated basal H_2O_2 production was found in patients with systemic sclerosis, systemic lupus erythematosus, mixed connective tissue disease and primary Raynaud's disease. For each group the trends in basal H_2O_2 production were paralleled by the degree of microvascular injury as assessed by serum factor VIII Rag levels. It is therefore interesting to speculate that neutrophil derived oxidants may contribute to the vascular pathology of connective tissue disease.

CHAPTER 1

Introduction

The neutrophil leukocyte is a principal effector cell of inflammation. However considerable argument exists as to whether the neutrophil is a brave soldier, defending the host from invasive micro-organisms or whether it might be causing considerable harm to the host as the result of such an encounter. With respect to the latter proposal activated neutrophils release highly reactive reduced oxygen species capable not only of killing micro-organisms but also causing significant injury to host cells.

The aim of this introduction is to discuss the origin and biological reactivity of these reduced oxygen species, their role in bacterial killing and the inflammatory response and finally their potential involvement in the pathogenesis of connective tissue disease.

The Biochemistry of Oxygen

Molecular oxygen (dioxygen) is a major biological oxidant. However, because of its unusual electronic structure it is unable to react directly with most organic compounds (Naqui and Chance 1986). Therefore, an activation step is usually required before dioxygen can function as a universal electron acceptor (Lamb and Elder 1931). At a cellular level this often involves complexation with or reduction by transition metals (Aust et al 1985). These, having unpaired electrons, make excellent catalysts of oxygen reduction. In fact, the activities of many oxidase and oxygenase enzymes are dependent on the transition metals associated with them. For example, cytochrome oxidase, a mitochondrial enzyme that catalyses the transfer of electrons from cytochrome C to oxygen (and in doing so regulates the synthesis of chemical energy in the cell), contains at least four metal atoms per functional unit (two copper and two iron). Reduced cytochrome C (the oxidase's physiological substrate) transfers electrons to an iron-copper (Fe-Cu) site on the enzyme which acts as an electron pool. The electrons are then shunted to a second Fe-Cu site to which oxygen has bound so reducing it to water (Naqui and Chance 1986). Partially reduced products of oxygen are generated during the electron transfer process or if electrons inadvertantly leak away from the active site of the enzyme. The participation of these in a number of normal and pathological states is becoming increasingly apparent (Cerutti 1985, McCord 1985).

The toxicity of oxygen has often been associated with the generation of its one electron reduction product, superoxide (O_2^-) (Freeman and Crapo 1982, Halliwell and Gutteridge 1984).

eg $M^{2+} + O_2 \dashrightarrow M^{3+} + O_2^-$ (Just 1907)

The reactivity of superoxide is however critically dependent upon its solvent. In aprotic conditions it is a more potent nucleophile and reducing agent than in aqueous solution (Fee and Valentine 1977). For this reason some investigators have dismissed the potential direct cytotoxicity of superoxide despite the presence of micro-environments within the cell which may be sufficiently aprotic to exploit its reactivity (eg cell and organelle membranes) (Bielski et al 1983).

In aqueous environments superoxide is much less reactive and predominantly undergoes dismutation to hydrogen peroxide (H_2O_2). Spontaneous dismutation occurs most efficiently in acid conditions whilst the enzyme catalysed dismutation (by superoxide dismutase) prevails at near neutral pH (Fridovich 1983).

eg $2O_2^- \xrightarrow{2H^+} H_2O_2 + O_2$ (George 1965)

Hydrogen peroxide is a powerful oxidant, yet like superoxide its direct reaction with many organic compounds occurs very slowly. However, it will react with transition elements (Fenton Reaction) to generate more reactive oxidants, for example the hydroxyl radical ($OH\cdot$)[1] (Posner 1953).

1. $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$ (Posner 1953)

2. $Fe^{2+} + H_2O_2 \longrightarrow Fe\,OH^{3+} + OH^-$ (Lamb 1931)

3. $Fe\,OH^{3+} \longrightarrow Fe\,O^{2+} + H^+$ (George 1965)

The formation of the hydroxyl radical is favoured in aqueous solution but ferryl ions ($Fe\,OH^{3+}$, $Fe\,O^{2+}$) may well be generated in aprotic media[2,3] (Walling 1982). In addition to its generation by Fenton type reactions OH· may also be produced by the reduction of H_2O_2 with superoxide (or other reducing agents). Transition elements allow this reaction to proceed in a very rapid manner (Haber-Weiss rection) (Kurimura 1968).

eg $O_2^- + M^{3+} \longrightarrow M^{2+} + O_2$

$O_2^- + HO_2\cdot \xrightarrow{H^+} O_2 + H_2O_2$

$H_2O_2 + M^{2+} \longrightarrow M^{3+} + \cdot OH + OH^-$

Of particular note is the ability of glutathione and ascorbate to substitute for O_2^- as reducing agents. These are present at far greater intracellular concentrations than that estimated for O_2^- and may therefore be physiologically more relevant. The nature of the physiological Haber-Weiss catalyst remains a subject of considerable controversy and will be discussed in detail in the next section.

Metal ions also facilitate the decomposition of hydrogen peroxide albeit at a much slower rate than the Fenton reaction. The ferric ion component of catalase promotes the two electron disproportionation of H_2O_2 to oxygen and water (Roos et al 1980).

eg $Fe^{3+} + H_2O_2 \longrightarrow Fe\,O^{3+} + H_2O$

$$Fe\ O^{3+} + H_2O_2 \dashrightarrow Fe^{3+} + O_2 + H_2O$$

Despite its lack of specificity (due to the generation of an $Fe\ O^{3+}$ complex which will also oxidase short chain alcohols and redox dyes) catalase is routinely employed to probe H_2O_2 involvement in biological oxidations (Goto et al 1970).

The Biological Activity of Reactive Oxygen Species

It is generally believed that the cytotoxic effects of reactive oxygen species lie in their ability to promote lipid peroxidation in cell and organelle membranes (Weiss and LoBuglio 1982). Recent evidence, however, suggests that they may also readily react with nucleic acids and proteins (Mello et al 1984, Woolf et al 1986).

The contribution of a particular species to cell injury has been investigated in vitro using free radical generating systems and target cells (Weiss and LoBuglio 1982) using inhibitors and free radical scavengers to confirm the nature of the participating species. For example, the inhibitory effect of superoxide dismutase indicates that O_2^- is in some way involved but leaves open the possibility that its metabolites may also play a role (McCord et al 1980). Sensitivity to catalase proves a role for H_2O_2 or more reactive species derived from it (Roos et al 1980). Involvement of $OH^{\bullet}$ radicals is indicated by the inhibitory effects of ethanol, mannitol and dimethylthiourea, whilst inhibition by tocopherol and carotenes points to the formation of lipid peroxides and singlet oxygen respectively (Flohe et al 1985).

The biological reactivity of cell derived oxidants have been partially dissected in a number of models co-incubating phagocytic cells with target cells in the presence of a suitable stimulant. However caution must be taken in interpreting results from these studies because firstly, phagocytes are often stimulated with agents that do not appear to have an in vivo correlate. For example, phorbol myristate acetate (PMA), is often employed by virtue of its ability to stimulate high levels of superoxide and H_2O_2 at the cell surface (De Chatelet et al 1976). Cell stimulation with PMA, however, leads to the release of specific but not azurophil granule constituents and therefore the generation of oxidants (ie $OH^{\bullet}$ radicals) that may not occur during cell stimulation in vivo (Winterbourn 1986). Secondly: the effectiveness of inhibitors or radical scavengers is dependent on their accessibility to the oxidant. In the system described above the proximity between phagocyte and target cell may be sufficiently close to prevent access to some inhibitors.

At a biochemical level oxidants readily interact with lipids, proteins and nucleic acids. Until recently it has been the widely held view that, the interaction between oxidants and membrane lipids underlie their cytotoxic effects (Dixit et al 1982). The generation of lipid peroxides is the key event in this destructive reaction and polyunsaturated fatty acids (PUFA) appear to be the primary target (Bielski et al 1983).

Lipid peroxidation occurs in several distinct phases (Tappel and Dillard 1981).

1) The removal of a hydrogen atom (abstraction) and formation of a lipid radical.

$$OH^{\bullet} + R - H \text{ ---------- } H_2O + R^{\bullet}$$

2) The reaction between oxygen and the newly formed lipid radical to generate lipid peroxy radical.

$$R^{\bullet} + O_2 \text{ ---------- } R - O_2^{\bullet}$$

3) The reaction between lipid peroxy radicals and neighbouring proteins, nucleic acids and lipids.

$$R - O_2^{\bullet} + R \text{ ---------- } R^{\bullet} + R\text{-}O_2H$$

In macromolecular terms the formation of lipid peroxides may alter the structural integrity of the cell membrane leading to increased membrane fluidity and an inability to maintain ion gradients (Mead 1976). Alternatively, the generation of aldehydes as lipid peroxide breakdown products can inhibit the activity of several important intracellular enzymes including glucose-6-phosphatase, adenylate cyclase and cytochrome P450 (Tappel 1981).

In contrast to their reactivity with lipids less is known about the effects of these oxidants upon other organic molecules and the consequences of such interactions. Proteins have recently received attention as targets for oxidative damage. Traditionally protein damage by free radicals has been attributed to the generation of lipid peroxides (Hochstein and Jain 1981). However, recent studies have demonstrated that reactive oxygen species can stimulate protein degradation by mechanisms independent of lipid peroxidation (Davies and Goldberg 1987). This can occur through two routes. Firstly by destabilising proteins through the formation of unstable carbon amino acid peroxides and secondly by enhancing enzymic

proteolysis. Increased proteolysis may occur by the direct free radical mediated activation of proteolytic enzymes or by increasing the proteins susceptibility to them through structural modifications (Nagy and Floyd 1984, Davies et al 1987). All amino acids are vulnerable to oxidative attack but tryptophan, histidine and cysteine are particularly susceptable (Davies at al 1987). Oxidative modification of amino acids may lead to changes in primary, secondary or tertiary protein structure (Davies and Delsignore 1987) and thereby unveil regions recognised by intracellular proteases.

The effects of oxygen derived free radicals upon nucleic acids and DNA are well illustrated by the toxic effects of the anti tumour antibiotic bleomycin (Burger et al 1981). Bleomycin interacts directly with DNA in the presence of Fe^{2+} to form of Bleomycin-DNA-iron complex. In the presence of oxygen the complex can act as a free radical generating system by reducing oxygen to superoxide and H_2O_2 to the hydroxyl radical. These can initiate nucleic acid scission and DNA fragmentation (Sauville et al 1978).

Neutrophil Activity and Inflammation

Inflammation is a protective and normal response to any stimulus that threatens the wellbeing of the host. When initiated by a single finite event the reaction is normally transient or self limiting, but should the stimulus persist or be self perpetuating, a sustained and potentially destructive reaction may ensue.

The cardinal signs of inflammation, heat, redness, swelling and pain have been recognised for over 2000 years and represent the complex microvascular changes that accompany injury or infection (Williams 1985). Reddening and heat are a consequence of increased regional blood flow due to local vasodilation whilst an increase in vascular permeability, secondary to the contraction of venular endothelial cells, leads to protein extravasation and swelling (oedema) (Moyno an Palade 1961, Williams 1985). Low molecular weight inflammatory mediators released from injured cells, plasma enzyme cascades and circulating phagocytes contribute sequentially to microvascular changes (Williams and Morley 1973, Hulstrom and Sveryo 1979, Mayno et al 1981, Williams and Jose 1981, Wemore and Williams 1981). The vasodilator responses to these mediators, can occur by a direct action on vascular smooth muscle or indirectly by stimulating the release of endothelium derived relaxing factors. Vasodilation can contribute significantly to the extravasation of protein by increasing post capillary hydrostatic pressure (Williams and Morely 1973, Furchgott 1981, Davies and Williams 1984, Palmer et al 1987).

The vascular components of inflammation, serve to increase the supply of oxygen, nutrients and circulating cells to the inflammatory focus. Polymorphonuclear leukocytes, particularly neutrophils, are amongst the first cells to arrive at the scene. They are consistent features of acute, sub acute and some chronic forms of inflammation and constitute the primary cellular defence against invasive micro-organisms (Wright 1981). Neutrophils develop in the bone marrow from pluripotential stem cells under the influence of a variety of cell derived factors (Queensbury and Gibrone 1980, Bagby et al 1981). About half the cells leaving the bone

marrow remain in the circulation, the remainder form a marginating pool and subsequently migrate to the extravascular spaces. Margination involves the orientation of neutrophils along the lumenal lining of blood vessels. In doing so they increase lamina blood flow and tend to adhere to endothelium (Lentek et al 1976). Adherence is initially an energy requiring process but it may be maintained passively (English and Cabig 1986). Changes in both neutrophil and endothelial cell are probably required to initiate the process. Stimulated neutrophils express antigenic glycoproteins on their cell surface which may be important since antibodies directed against these inhibit both adherence and adherence related functions (Anderson et al 1985, Anderson et al 1986, Pham-Hull et al 1987, Lewinsohn, et al 1987).Products from neutrophil specific granules (eg lactoferrin) have been implicated in the adherence process (Oseas et al 1981, Babior et al 1981). Butcher et al have suggested that following degranulation at an inflammatory centre the antigenic glycoproteins located in the specific granules are translocated to the cell surface where they are able to promote adherence.

Endothelial cells can mediate neutrophil adherence both non specifically by releasing pro-adhesive factors like fibronectin and specifically through the directed synthesis of interleukin-1 dependent binding sites and cell associated platelet activating factor (Pearson er al 1979, Peters et al 1986, McIntyre et al 1986, Butcher et al 1986).

The biochemical basis of adherence is uncertain but the formation of ionic bonds seems likely in view of the alterations in net cell surface charge that occur when agents stimulate the process (Gallin et al 1975).

Under electron microscopy the passage of neutrophils through endothelium occurs by a non-destructive amoeboid process (Florey and Grant 1961). At some point therefore adherence must be terminated and active movement initiated. The orientated locomotion of neutrophils requires their exposure to a concentration gradient of a specific stimulus - (chemotactic factor) (McCutcheon 1946). Cells undergoing chemotaxis fixed by glutaraldehyde undertake a polarized morphology (Haston and Shields 1985). This is thought to represent the longitudinal waves of cytoplasmic contraction that accompany undirectional locomotion (Shields and Haston 1985). During chemotaxis the neutrophils cellular recognition units (receptors) are orientated towards the leading edge of the cell to optimise the detection of chemo-attractant and ensure that the origin of locomotion lies at the front of the cell. Unlike adherence, chemotaxis requires intact microfilaments and microtubules (Malech er al 1977). Agents that interfere with microtubules (eg Colchicine) prevent pseudopod formation and inhibit directed migration in vitro. Whilst agents that affect microfilaments (eg cytochalasin B) also abolish directed migration but without effecting the orientation of intracellular structures (Malech et al 1977).

The biochemical basis of chemotaxis has been investigated by several groups (Taylor and Condeelis 1979, Grinstein et al 1986, Shaafi and Molski 1987). A common effect of chemotacic agents is to activate the sodium/proton pump and thereby increase intracellular pH (pHi). Amiloride and its derivatives inhibit cell alkalinisation and likewise neutrophil chemotaxis in vitro (Taylor and Condeelis 1979). Changes in pHi may

therefore be important in regulating cytoskeletal organisation and hence various aspects of cell motility.

Under the influence of chemotactic factors neutrophils emigrate from the circulation into the extravascular spaces and towards the origin of inflammation. Upon contact with the offending particle, pseudopodia are extended to surround it and fuse at the distal side so that the particle becomes enclosed within a phagosome (Stossel 1974). Although neutrophils are able to engulf (phagocytose) seemingly inert particles (eg latex beads) ingestion is markedly inhanced by two serum factors, the third component of complement and immunoglobulin (C3b, IgG) (Wright and Douglas 1903). These proteins coat (opsonise) the particle making it more palatable for the neutrophil. Both C3b and immunoglobulin promote the maximal rate of particle ingestion but only the immunoglobulin (IgG) increases the cells affinity for the particle (Stossel 1973).

The phagososome consists of an inverted plasma membrane enclosing cytoplasm. Unlike mononuclear phagocytes neutrophils are unable to synthesize new plasma membrane and are therefore limited in the number of particles they can engulf (Werb and Cohn 1972). As the phagosome forms, primary (azurophil) and secondly granules (specific) which contain proteolytic enzymes move rapidly towards it fuse, and then disappear from the cytoplasm (hence degranulation) (Hirsch and Cohn 1960). The formation of intact phagolysosomes is essential for the host to isolate the destructive constituents within them. Under some circumstances, however, extracellular discharge may occur. These include the suicide attack of crystals and the attempted phagocytosis of tissue bound particles

(Frustrated phagocytosis). In these situations considerable extracellular injury may well occur (Palmbad 1984).

Neutrophil-derived Oxidants

In addition to generating reactive oxygen species as byproducts of cellular metabolism, phagocytic cells have evolved a unique superoxide generating system for the specific killing of invasive micro-organisms (Babior 1977). Activation of the enzyme responsible, a reduced pyridine (NADPH) oxidase, is associated with the series of metabolic events that accompany phagocytosis or stimulation with non particulate agents (De Chatelet 1978). These include a marked increase in oxygen consumption, increased glucose phosphate breakdown via the hexose monophosphate shunt and the generation of superoxide and hydrogen peroxide (Baldridge and Gerald 1933, Sbarra and Karnovsky 1959, Iyer et al 1961, Curnutte and Babior 1974). NADPH oxidase which appears to be a transmembrane protein, catalyses the transfer of an electron from the reduced pyridine nucleotide (NADPH) to molecular oxygen so reducing it to superoxide (Iyer and Quastel 1963). The supply of electrons by NADPH to the system is maintained when the cytosolic ratio of NADP:NADPH is sufficiently high to stimulate the hexose monophosphate shunt (Sbarra and Karnovsky 1959).

That the oxidase is associated with the cell membrane has been conclusively demonstrated by several workers (Babior et al 1976, Goldstein et al 1977, Dewald et al 1979, Babior et al 1981). Of particular importance to their conclusions was the ability to account for all extra oxygen consumed during cell activation as superoxide in cytochalasin B paralysed neutrophils.

Cytochalasin B prevents phagocytosis and therefore accessibility of superoxide to intracellular dismutase enzymes. Quantitative recovery of $O_2^{-\cdot}$ would be unlikely if it were being released into the cytoplasm because under these circumstances the superoxide dismutase enzymes of the neutrophil would almost certainly destroy at least part of it before it could diffuse out through the plasma membrane (Salin and McCord 1974)

The passage of electrons between NADPH and oxygen is mediated by a multicomponent electron transport chain which includes a low potential cytochrome and an FAD containing flavoprotein (Babior and Kipnes 1977, Segal and Jones 1978). Activation of the enzyme probably involves a phosphorylation step prior to or at the level of the flavoprotein. This enables it to accept an electron from NADPH and subsequently transfer it to the cytochrome which has a sufficiently low redox potential (-245 mV) to directly reduce oxygen to superoxide (Gabig et al 1981, Cunningham et al 1982, Bellavite et al 1985, Hurst 1987) (Fig 1).

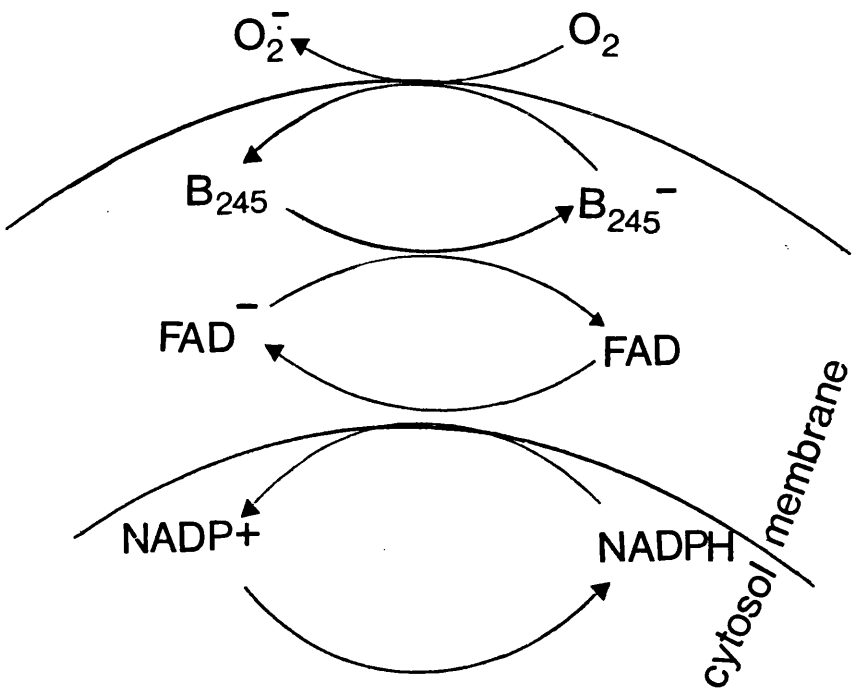
The importance of these electron transfer components in oxygen reduction has been confirmed in studies on patients with x-linked chronic granulomatous disease. Neutrophils from these patients lack or have an abnormal b type cytochrome and are unable to mediate the oxidative killing of bacteria (Segal et al 1978).

That phagocytes produce potentially toxic metabolites of oxygen was first demonstrated when Iyer measured H_2O_2 in the medium surrounding stimulated neutrophils (Iyer et al 1961). In addition to H_2O_2 it has been predicted that neutrophils are able to produce more reactive species, for

example singlet oxygen ($^1O_2$) and the hydroxyl radical ($OH^\cdot$). Conclusive demonstration of these species is imperative in view of their capacity to initiate widespread tissue damage. However, their highly reactive nature has hampered detection and their physiological relevance remains equivocal.

In principle $OH^\cdot$ radicals can be generated by the metal catalysed reduction of H_2O_2 (Winterbourn 1979). Iron-binding proteins have received considerable attention as putative physiological catalysts. Iron bound to transferrin (the plasma iron transporter) is only poorly active at promoting $OH^\cdot$ production or lipid peroxidation (a functional assay for $OH^\cdot$). However, at low pH the efficacy of transferrin as a Haber-Weiss catalyst increases and may well be of relevance in the phagolysosomal environment (Gutteridge et al 1981, Subak and Sharpe 1981, Motohaski and Mori 1983). Iron from ferritin, the intracellular iron storage protein is an effective catalyst of $OH^\cdot$ radical production (Blemond et al 1984). Free iron is required and dissociation from the protein complex is achieved by a superoxide dependent reductive mobilization or non specific damage by peroxides (Biemond et al 1986). Another source of intracellular iron is that bound lactoferrin. Lactoferrin is confined to the neutrophil specific granule and therefore ideally situated for generating $OH^\cdot$ radicals within the phagolysosome. It is however only about 25% iron saturated and a relatively poor catalyst of $OH^\cdot$ production (Sutton 1985).

Figure 1 THE ELECTRON TRANSPORT CHAIN OF NADPH OXIDASE



Thus far, all the biologically relevant extracellular iron chelates examined have low efficiencies as Haber-Weiss catalysts. Furthermore in order for a Haber-Weiss reaction to proceed it must compete favourably with other reactions using H_2O_2. Many stimuli that induce superoxide production also cause the release of lysosomal myeloperoxidase (MPO) (Baggiolini and Dewald 1984). MPO, a major constituent of the azurophil granule, catalyses the halogenation of H_2O_2 into potent bactericidal agents (eg hypochlorous acid) and possesses catalase and peroxidase activity (Klebanoff 1982, Winterbourn et al 1985, Iwamoto et al 1987). By utilising available H_2O_2, MPO could inhibit $OH^{\bullet}$ radical formation in conditions where release of azurophil constituents occurs (Winterbourn 1986).

The Bactericidal Activity of Neutrophils

Phagocytosed micro-organisms are killed by high concentrations of oxidants generated locally within the phagosomal membrane and ultimately degraded by proteolysis following lysosomal degranulation (Stossel 1974 and Babior 1978). Iyer et al (1961) were first to propose that H_2O_2 constituted an important bactericidal agent. Unlike $O_2^{\bullet}$, H_2O_2 possesses intrinsic antimicrobial activity, the potency of which can be enhanced dramatically by myeloperoxidase (MPO) (Rosen and Klebanoff 1979). This haemoprotein enzyme, (Bainton and Farquhar 1968) catalyses the halogenation of H_2O_2 to hypohalous acids and in particular hypochlorous acid (Harrison and Schultz 1976) (1). The cytotoxic activity of hypochlorous acid involves oxidation and halogenation of microbial cell walls. Alternatively the formation of chloramines can occur when hypochlorous acid reacts with

free amine groups (2) (Grimsham et al 1984). These long-acting metabolites can oxidise sulphydryl groups and generate cytotoxic aldelydes (Fantone and Ward 1985). MPO derived oxidants play an important but not essential role in microbial killing. Unlike patients, with chronic granulomatosus disease, those with MPO defficiency do not suffer the constant possibility of contracting life threatening infections (Salmon et al 1970). Bacterial killing in MPO defficient patients is delayed but none the less complete (Klebanoff and Hamon 1972). Thus, other oxygen dependent killing mechanisms are probably equally important as those dependent on MPO Klebanoff (1982) has recently identified an antimicrobial system in which H_2O_2 is reduced by Fe^{2+} to generate $OH^{\cdot}$ radicals (3). In the presence of iodide ions, hypoiodous acid and iodide radicals are formed that are capable of iodinating target molecules with lethal effects.

$$H_2O_2 + Cl^- \xrightarrow{MPO} O\,Cl^- + H_2O \quad (1)$$

$$O\,Cl^- + RNH_2 \longrightarrow RNHCl + HO^- \quad (2)$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + HO^- + HO^{\cdot} \quad (3)$$

$$HO^{\cdot} + I^- \longrightarrow HO^- + I^{\cdot}$$

$$I^{\cdot} + H_2O_2 \longrightarrow HOI + HO^{\cdot}$$

Intracellular and Extracellular Antioxidants

The intrinsic reactivity of superoxide and its metabolites constitutes a great threat to cellular integrity. Much of the tissue damage attributed to oxidants is thought to be mediated by $OH^{\cdot}$ radicals, (Little and O'brien 1968) due to their ability to peroxidise membrane lipids and initiate lipid peroxy radical chain reactions (Mead 1976).

Iron is involved in various aspects of oxidative injury through its ability to catalyse the Haber-Weiss reaction and by facilitating the decomposition of lipid peroxides. Iron bound to extracellular transport proteins is a poor catalyst for $OH^{\cdot}$ production and under normal conditions free iron levels are extremely low (Halliwell and Gutteridge 1986). For this reason iron binding proteins can function as extracellular antioxidants by preventing both the formation and propagation of active oxygen species (Fantone and Ward 1985).

Additional extracellular protection from oxidants is afforded by ceruloplasmin (Denko 1979). This α_2 globulin is produced by the liver in response to injury or infection (acute phase response). Ceruloplasmin is a putative copper transport protein though this function is hard to demonstrate. However, it also exhibits powerful scavenging activity toward $OH^{\cdot}$, $^1O^2$ and O_2^- (Goldstein et al 1978). In addition ceruloplasmin possess ferroxidase activity and promotes the oxidation of Fe^{2+} to Fe^{3+}. This depletes the availability of the redox-metal for Haber-Weiss reactions, and enhances the breakdown of H_2O_2 to water (Lystad 1981, Gutteridge 1983).

The presence of an intracellular mobile iron pool (iron bound to ferritin) may be important in promoting oxidative damage. Intra-cellular anti-oxidants are provided largely by specific enzyme systems which promote the conversion of reactive oxidants to more inert species. Superoxide dismutases (SOD) catalyse the dismutation of O_2^- to H_2O_2 (McCord and Fridovich 1969). Two forms of the enzyme are present in eucaryotes, a copper-zinc SOD in the cytosol and a manganese form in mitochondria. Catalase and glutathione peroxidase catalyse the breakdown of H_2O_2 to

water (Chance et al 1979, Roos et al 1980). The anti-oxidant activity of glutathione peroxidase is coupled to the intracellular concentration of glutathione, glutathione reductase and NADPH. Glutathione reductase maintains the supply of reduced glutathione by catalysing the transfer of electrons to it from NADPH. Glutathione peroxidase can then catalyse the glutathione dependent reduction of H_2O_2 to water (Meister and Anderson 1983). In contrast to glutathione peroxidase, catalase requires no additional cofactors in order to convert H_2O_2 to water.

Vitamin E (α tocopherol) represents an important cellular defence against lipid peroxidation (Lucy 1972). It is located in biomembranes and has the capacity to scavenge O_2^-, $OH^\bullet$, $^1O_2$ and the peroxy lipid radical (R - $O^\bullet$). The efficiency of vitamin E as a scavenger is partly dependent upon the presence of ascorbic acid (vitamin C). Vitamin E can accept electrons from peroxy lipid radicals and subsequently transfer them to vitamin C which in turn can transfer electrons to NADPH (see below) (Palker et al 1979).

$$\begin{array}{cccccc} \text{LIPID-OH} & & \text{VIT E}^\bullet & & \text{VIT C} & & \text{NADP}^+ \\ & \rightleftharpoons & & \rightleftharpoons & & \rightleftharpoons & \\ \text{LIPID-O}^\bullet & & \text{VIT E} & & \text{VIT C}^\bullet & & \text{NADPH} \end{array}$$

Reactive Oxygen Species and Cell Death

A delicate balance exists between the cellular systems that generate oxidants and those that maintain antioxidant defence mechanisms. Should the balance be in favour of the former, irreversible cellular injury and death soon follow. The nature of the final cytotoxic insult is as yet

unknown. Several workers have suggested that alterations in Ca^{2+} homeostasis may be important (Thor et al 1982, Orrenius 1985, Scherer and Deamer 1986). Oxidative stress impairs the function of calcium ATPase enzymes. This prevents the extrusion of intracellular calcium and its uptake by mitochondria and sarcoplasmic reticulum. The increase in cytosolic calcium that results activates protease, kinase and phospholipase enzymes and causes cytoskeletal and membrane alterations.

That the process is reversible by dithiothreitol indicates that is is the oxidation of thiol groups on the Ca^{+2} ATPase enzyme that is the important event (Ornius 1985). The maintenance of free intracellular thiol groups is the responsibility of glutathione (Meister and Anderson 1983). During periods of oxidative stress intracellular glutathione is depleted because its conversion from oxidised (GSSG) to reduced (GSH) form by glutathione reductase is slow (Thor et al 1982). The build up of oxidased glutathione encourages the cell to actively secrete it thus depleting the total cellular pool of reduced glutathione. It is this depletion that leads to increased protein thiol oxidation and its consequences on calcium and cell homeostasis (Ornius 1985).

Reactive Oxygen Species as Inflammatory Modulators

Although neutrophil derived oxidants can provoke inflammation by direct injury to bystander cells, they can also influence the course of an inflammatory reaction in more subtle ways. A bidirectional control of chemotaxis by reactive oxygen species has recently been reported. Superoxide contributes to the chemotactic activity of C5a and leukotriene B_4 (LTB_4) and can interact with serum lipids to generate a factor with specific chemotactic activity towards neutrophils (McCord 1980, Flohe et al 1984). H_2O_2 and MPO derived oxidants on the other hand have been implicated in the inactivation of chemotactic factors (Anderson and Jones 1982, Clarke 1982). H_2O_2 may also play a role in modulating levels of LTB_4. Patients with chronic granulomatous disease generate normal levels of LTB_4 and LTC_4 compared to normal subjects and those with MPO deficiency. However, levels remain elevated in the CGD patients whilst falling rapidly in the other two groups. Thus, oxidative inactivation may be an important mechanism for regulating the activity of leukotrienes (Henderson and Klebanoff 1983).

An important effect of oxygen derived free radicals is their ability to generate aggregates from monomeric IgG (Jasin 1983, Lunec 1984, Wickens and Dormandy 1984). Aggregated IgG is a powerful stimulus for neutrophils whilst the monomer is inactive (Goldstein et al 1975, Gale et al 1985). By cross linking Fc receptors aggregated IgG can stimulate the release of lysosomal enzyme, reactive oxygen species and lipid mediators (Frey et al 1986). Extracellular release of lysosomal protease enzymes may cause significant cellular injury (Henson 1971). Of relevance to this thesis is the ability of certain neutrophil proteases to digest key structural elements

of connective tissue and cause the detachment, increased permeability and even lysis of vascular endothelial cells (Henson and Johnston 1987). Elastase in particular has been implicated in neutrophil mediated injury to endothelial cells in vitro (Smedly et al 1986). Under normal circumstances negligible elastase activity is detectable in serum. First, because phagolysosome formation is an extremely efficient process and lysosomal proteases are only released when neutrophils attempt to engulf tissue bound particles or crystals (Henson 1971) and secondly because circulating antiproteases bind and inactivate the proteases (Ohlsson 1978). Under conditions of oxidative stress, circulating protease inhibitors are inactivated by oxidative modification (Carp and Janoff 1979, 1980). The absence of antiproteases in the extravascular compartment may facilitate neutrophil migration, to the inflammatory focus, but within the blood stream considerable microvascular injury may occur (Henson and Johnston 1987).

In spite of their ability to promote the release and activity of lysosomal proteases recent reports suggest that oxidants can also inactivate lysosomal enzymes (Vissers and Winterbourn 1987). Thus an extremely complex relationship between the pro and potentially anti-inflammatory effect of reactive oxygen species exists. The balance of effects may serve to regulate or limit the spread of inflammation around the inflammatory focus.

Molecular Mechanisms of Neutrophil Activation

Neutrophil activation, be it chemotaxis, phagocytosis or oxidative metabolism, requires the binding of appropriate stimuli (or ligands) to

specific membrane glycoproteins or receptors (Henson 1976). Neutrophils express receptors for the complement components (C3b and C5a) (Kay et al 1979) immunoglobulin (IgG, IgA) (Mantovani 1975, Henson 1976) bacterial peptides (Schiffman et al 1975) and the products or arachidonic acid metabolism (eg LTB_4) (Goldman and Goetzel 1984).

The ligand-receptor interaction leads to the generation of intracellular chemical signals (second messengers) which translate the binding signal into functional cellular activity (Berridge 1981). The nature of each chemical signal varies not only with the stimulus employed but probably also with the ultimate response. Second messengers modulate cellular activity by binding to and activating kinase enzymes. Through a subsequent phosphorylation step these enzymes control the local apparatus regulating a particular cell response (see Table 1) (Greengard 1978).

GTP binding proteins (G proteins) couple receptor glycoproteins to the enzyme systems which generate second messengers (Rodbell 1980, Dohlman et al 1987). G proteins have several common features: they possess three subunits (α, B and γ), a GTP binding site, and intrinsic GTPase activity (Litosch 1987). Thus far the most studied receptor - 2nd messenger - effector system is that which couples adrenergic receptors to adenylate cyclase and cAMP production (Gilman 1984). Adenylate cyclase activity may be regulated by ligands binding to B_2 or α'_2 receptors (Lefkowitz and Hoffman 1981). B_2 receptor stimulation results in the activation of adenylate cyclase through a GTP binding protein (G_s). G_s, like other proteins, contains α, B and γ subunits. Ligands acting at B adrenergic receptors stimulate the α subunit of G_s to bind GTP and then promote

its subsequent release from the $B\gamma$ complex. The free α subunit associates with and activates the catalytic site of adenylate cyclase thereby stimulating a rise in intracellular cyclic adenosine monophosphate (cAMP) (Northup et al 1983). The inherent GTPase activity of the α subunit results in the loss of GTP from it, and its dissociation from the catalytic site of the enzyme (thus terminating enzyme activity).

Adenylate cyclase activity is also under the control of ligands which stimulate α_2 receptors. These agents inhibit enzyme activity through another G protein (Gi) (Neer et al 1984). Gi differs from G_s in that it can be inactivated by pertussis toxin (Becker et al 1986). The ligand promoted binding of GTP to Gi results in the release of $B\gamma$ units from the Gi complex. These mop up the free α units generated by agents which stimulate adenylate cyclase through G_s and thereby inhibit the activation of the enzyme (Gilman 1984).

In the neutrophil a rise in intracellular cyclic AMP levels leads to the inhibition of several aspects of cell activity ie cAMP is an inhibitory second messenger (Ignarro et al 1976). Ligands which stimulate functional neutrophil activity (eg FMLP and aggregated IgG) utilise diacylglycerol (DAG) and calcium as second messengers (Cockroft et al 1981, Suzuki et al 1985). Diacylglycerol levels are transiently raised following the ligand stimulated activation of phosphatidylinositol specific phospholipase C (PLC) (Hirasawa and Nishizuka 1985). That pertussus toxin can inhibit the FMLP stimulated rise in DAG indicates that G proteins (similar to Gi) may regulate the activity of this enzyme (Bradford and Rubin 1985, Cockroft and Gomperts 1985, Dickey et al 1987).

With regard to the activation of neutrophil NADPH oxidase and the stimulation of superoxide production, the phosphorylation of a regulatory component of the enzyme appears to be essential for its activity (Cross and Jones 1986). Agents which stimulate a rise in DAG may well mediate the phosphorylation step through protein kinase C (PKC) (Sharkey et al 1984). The role of PKC in neutrophil activation has been investigated using a group of experimental stimuli, the phorbol esters (eg phorbol myristate acetate - PMA). PMA, a potent stimulus of superoxide production bypasses the cell surface to interact directly with the cytoplasmic aporeceptor enzyme, PKC. In doing so the enzyme's affinity for calcium ions is increased and its translocation from the cytosolic to the membrane fraction facilitated (Niedel et al 1983). Here in the presence of phosphatidylserine it becomes fully active and can phosphorylate a variety of proteins. Alternatively, PKC can undergo proteolysis to a calcium-and phospholipid-independent kinase (PKM) which is subsequently released into the cytosol. The function of PKM is as yet unknown (Mellonie et al 1986).

The target substrate for PKC has been investigated by comparing the phosphorylated products of normal neutrophils stimulated with PMA with those from patients with chronic granulomatous disease (CGD) (Hayakawa et al 1986). Of interest, was the discovery that CGD patients lacked phosphorylated products in the 44-48 kD protein band. Furthermore Okamura et al (1984) have demonstrated in partially purified enzyme preparations a 46kD substrate for PKC: an important finding in the light of the missing phosphorylated CGD proteins.

Neutrophils stimulated with agents that raise DAG levels and activate NADPH oxidase (eg FMLP) demonstrate membrane phosphorylation patterns similar to PMA: ie they appear to be acting through protein kinase C (Schneider et al 1981, Oshtyzuka et al 1987). Furthermore, synthetic analogues of DAG are able to stimulate both NADPH oxidase and PKC translocation in neutrophils. Therefore DAG appears to be the physiological activator for PKC (Kikkawa et al 1983).

The importance of calcium ions (Ca^{2+}) as a second messenger of cell activation has been inferred from several observations.

1. Neutrophil stimulation with some ligands (eg FMLP and LTB_4) results in the mobilization of calcium from intracellular stores (White et al 1983, Naccache et al 1984).
2. Extracellular calcium can control the magnitude of a cellular response to some ligands (Smolen et al 1981).
3. Agents which mediate calcium influx are themselves stimulatory to cells (eg calcium ionophore) (Goldstein et al 1974).

Smolen et al (1981) have examined the contribution of calcium to the neutrophil oxidative response. Using the intracellular calcium antagonist TMB-8 and an extracellular calcium chelator, (EGTA) he demonstrated that although extracellular calcium was required to ilicit a full magnitude repsonse to some stimuli, it was the mobilisation of intracellular calcium that was pre-requisite for NADPH oxidase activity (Smolen et al 1981).

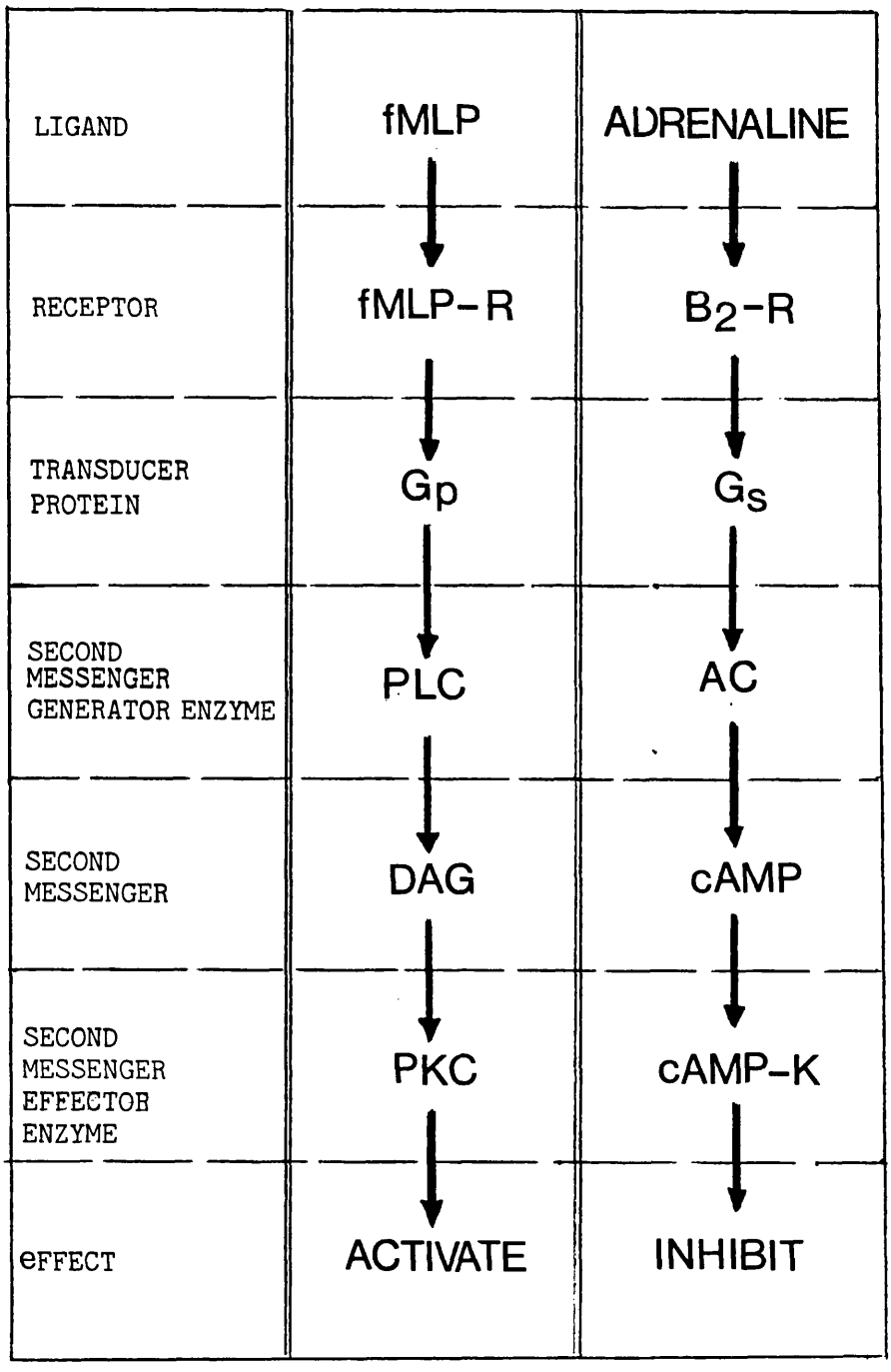
When neutrophil stimulation is accompanied by the activation of PLC, intracellular calcium mobilization is probably mediated by inositol 145 triphosphate (IP_3) (Streb et al 1983). IP_3 is generated concomitantly with DAG by the action of PLC on phosphatidylinositol biphosphate. Other mechanisms may also be involved in raising intracellular Ca^{2+} levels. For example, IgG (aggregated) increases intracellular calcium by cross linking Fc receptors (Anderson et al 1986). Neutrophil stimulation by IgG is not accompanied by the breakdown of phosphatidylinositol and therfore it is unlikely that IP_3 plays a role in calcium mobilization by this agent.

Agents which elevate intracellular calcium may activate NADPH oxidase by a phosphorylation step involving calmodulin dependent kinase enzymes. Certainly addition of calmodulin to preparations of NADPH oxidase stimulates its enzymic activity and calmodulin inhibitors reduce the oxidative response of neutrophils to soluble stimuli (Alobaidi and Jones 1982). FMLP and LTB_4 can in theory increase intracellular calcium through IP_3. Therefore they may be able to activate NADPH oxidase by routes other than protein kinase C (ie through calmodulin). With regard to this it is of special interest to find that oxidative responses to FMLP are only poorly inhibited by selective PKC inhibitors (eg the isoquinoline sulphonamides) (Gerrard et al 1987). Inhibitors of phospholipase A_2 (PLA_2) however can inhibit the oxidative response to FMLP (and opsonized zymosan) (Smolen and Weissman 1980). PLA_2, a calmodulin dependent enzyme cleaves arachidonic acid from membrane phosphatidylcholine (Daque et al 1986). Although arachidonic acid itself is able to stimulate NADPH oxidase in intact cells, its effects are again susceptible to inhibitors of PLA_2 (Maridonneau and Tauber 1986). This

indicates that activation of PLA_2 per se and not the products of its catalytic activity may to be important in activating neutrophil NADPH oxidase. How PLA_2 can achieve this remains a mystery but the changes in membrane fluidity that accompany its activity may well be important (Sakata et al 1987). Certainly the increased membrane fluidity brought about by anionic detergents appear to be responsible for NADPH oxidase activation by these agents (Cohen and Chovaniec 1978).

Table 1

Signal transduction pathways in neutrophils stimulated by FMLP/adrenaline.



Low Molecular Weight Lipid Mediators of Inflammation

The activation of phospholipase C (PLC) and subsequent breakdown of phosphatidylinositol biphosphate (PIP_2) is associated with the series of intracellular events through which ligand-receptor interaction influences cellular activity (Takenawa et al 1985, Hurst 1987). In addition to activating PLC some ligands initiate phospholipid breakdown through a phospholipase A_2 (PLA_2) dependent pathway (Bormann et al 1984, Weissman 1987). PLA_2, a calcium/calmodulin dependent enzyme, catalyses the liberation of arachidonic acid from membrane phosphatidylcholine (Walsh et al 1981, 1983; Engelberger 1984). The availability of free (unesterfied) arachidonic acid has important implications for inflammation. Its oxidative metabolism results in the generation of a series of potent inflammatory mediators, the eicosanoids. In this repect two enzyme pathways are important. The first, via cyclo-oxygenase leads to the generation of stable prostaglandins (eg PGE_2 and F_2), the unstable thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) (Weissman 1983).

The prostaglandins are short lived molecules, being rapidly converted to inactive metabolites. PGE_2 for example has a half life in the circulation of about 30 seconds before being metabolised by oxidation to 15 keto PGE_2. Likewise, prostacyclin is converted to 6 keto $PGF_{1\alpha}$ with an almost complete loss of biological activity (Ferreira and Vane 1967).

Prostaglandins E_2 and I_2 contribute to the microvascular changes in inflammation. They are vasodilators in their own right and potentiate the vasodilation and increased vascular permeability produced by other mediators (Wedmore and Williams 1981). Prostacyclin and thromboxane

A_2 are important regulators of platelet activation and may contribute to inflammation by modulating the release of platelet derived products (Robinson 1985).

Free arachidonic acid can also be oxidised by the lipoxygenase pathway to mono and dihydroxyeicosatetraenoic acids (HETE'S). The 5 lipoxygenation of arachidonic acid to leukotriene A_4 (LTA_4) and leukotriene B_4 (LTB_4) is the major route of arachidonic acid metabolism in neutrophils whilst monocytes predominantly form the thionylpeptide derivatives of LTA_4 ie LTC_4 and LTD_4 (Scott et al 1982, Godfrey et al 1987, Fitzharris et al 1987).

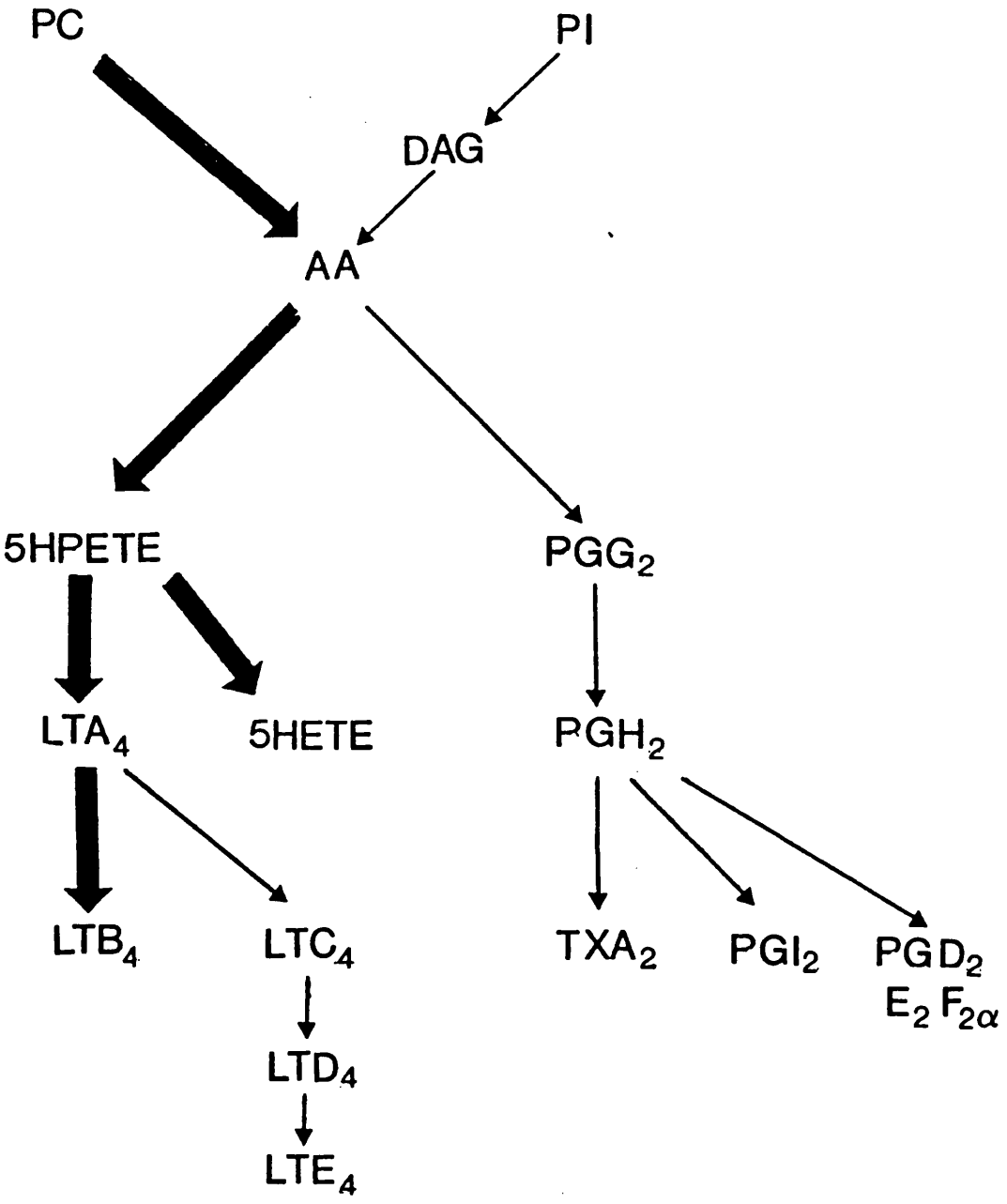
The leukotrienes are undoubtedly important mediators of inflammation. LTB_4 is a potent chemotactic agent towards neutrophils and monocytes and may act synergistically with vasodilator prostaglandins to increase vascular permeability (Ford-Hutchinson et al 1980). Furthermore LTB_4 stimulates neutrophil chemotaxis enzyme release and superoxide production (Smith 1987). The thionylpeptide derivatives of LTA_4 are powerful arterioconstrictors but also promote plasma leakage at postcapillary venules and stimulate the adhesion of neutrophils to vascular endothelium (Dahlen et al 1981, McIntyre et al 1986).

The inhibition of eicosanoid synthesis forms a major rationale in the treatment of inflammatory disorders. Corticosteroid drugs (eg hydrocortisone) are probably the most effective therapeutic agents for the treatment of chronic inflammatory states (Dannenberg 1979). Their activity is partly dependent upon the directed synthesis, through the

classical steroid pathway, of the antiphospholipase A_2 protein lipocortin (Hirata et al 1980, Blackwell et al 1980). The antiphospholipase activity of lipocortin is regulated by its phosphorylation state. It is a substrate for tyrosine and cAMP dependent (PKA) kinases and possibly protein kinase C (Hirata et al 1984, Pepinsky and Sinclair 1986). The phosphorylation of lipocortin results in a loss of antiphospholipase activity and is associated with an increase in arachidonic acid release (Hirata 1981). The mode of inhibition by lipocortin probably involves a reversible binding to and inactivation of the enzyme (Hirata 1981). Recently, however, it has been proposed that the effects of lipocortin may also be the result of sequestering the phospholipid substrate (Davidson et al 1987).

Human lipocortin has recently been cloned expressed and sequenced (Wallner et al 1986). The gene was expressed in E-coli and resulted in the generation of a 37kD protein with antiphospholipase activity (lipocortin 1). The cloned product can be phosphorylated by PKA and tyrosine kinase with loss of activity and appears to possess the full biological activity of its physiologial counterpart (Wallner et al 1986).

Figure 2 - PATHWAYS OF PHOSPHOLIPID METABOLISM IN PHAGOCYTIC CELLS.



Systemic Sclerosis (SS)

Systemic sclerosis is a disease characterised by widespread tissue fibrosis (Rodnan 1979). Although immunological abnormalities are present, their role in the pathogenesis of the disease is less well understood than for example in systemic lupus erythematosus. Recently, autoantibody profiles have been employed in a clinical setting to characterise SS variants (Riboldi et al 1985). The presence of antibodies to Scl 70 is a characteristic of patients with diffuse skin fibrosis whilst the anticentromere antibody is commonly found in patients with more limited skin involvement, and particularly those patients with CREST syndrome (calcinosis, Raynaud's, esophageal dysmotility, sclerodactyly and telangiectasia) (Tan et al 1980, McCarty et al 1983).

The most striking feature of SS is the often widespread hardening and thickening of the skin (Rodnan 1979). The extent of skin involvement may be limited to the extremities, especially the hand (sclerodactyly) or have extended to the forearm (proximal scleroderma) or the whole trunk (diffuse scleroderma). The abnormal fibrotic reaction in the skin is the result of increased synthesis of subcutaneous connective tissue elements, particularly collagen, on a background of reduced collagenase activity (LeRoy 1972, Brady 1975). This fibrosis is usually accompanied by the histological signs of inflammation, ie mononuclear leukocyte and plasma cell infiltration (D'angelo et al 1969, Jimerez 1983). Their proximity to the fibroblasts in SS skin lesions suggests that they may regulate collagen production. Indeed, factors released from normal stimulated mononuclear

cells (especially T-cells) inhibit collagen synthesis by normal and scleroderma fibroblasts in vitro (McArthur er al 1982, Jumenez 1983).

Intimal fibrosis of the arterioles and small arteries are the hallmark of the histopathology of SS and may constitute the primary lesion (Rodnan et al 1980). Evidence of microvascular abnormalities are considerable. Clinically, non pitting oedema, telangiectatic blood vessels and the prevalence of Raynaud's phenomenon (intense peripheral vasospasm in response to exogenous stimuli) are indicative of microvascular involvement (Ruggieri and LeRoy 1986). Histologically, intimal proliferation of the small arteries and arterioles are apparent with the loss of true endothelium and its replacement with cells derived from smooth muscle. Lumenal narrowing and occlusion follow with the degeneration of associated capillaries (Sinclair et al 1976, Kahaleh et al 1979).

Platelet abnormalities have been described which may be relevant to the development of fibrosis. Increased levels of platelet products (eg B thromboglobulin) and circulating aggregates indicate platelet activation (Kahaleh et al). Activated platelets release growth factors capable of stimulating fibroblast proliferation and therefore increased collagen synthesis (Castar et al 1979). Exposure to sub-endothelial collagen is a major stimulus for platelet activation (Baumgartner 1973). This can only occur following endothelial cell damage and leaves open the question as to what causes the initial lesion to endothelial cells? An exciting discovery by Kahaleh and others was that SS serum contained a factor cytotoxic to endothelial cells (Kahaleh et al 1979, Cohen et al 1983). Analysis of the toxin revealed that it was an oxidised lipoprotein and that its

development was dependent upon pre-exposure to oxidants in vivo (Blake et al 1984). In view of this it is of special interest that the toxicity of bleomycin (an antitumour antibiotic) can manifest itself in a scleroderma-like syndrome (Finch et al 1980, Moseley et al 1986). Bleomycin associate with free iron and in the presence of oxygen forms a free radical generation system capable of initiating lipid peroxidation (Kameda et al 1979, Lin et al 1980).

Neutrophils represent a major source of extracellular oxidants. Several workers have reported enhanced oxidative metabolism by these cells in scleroderma (Kovacs et al 1986, Czijak et al 1987, Maslen et al 1987). It may well be that neutrophil derived oxidants are responsible for damage to endothelial cells either directly, or indirectly through the generation of cytotoxic factors.

Systemic Lupus Erythematos us (SLE)

SLE, like scleroderma is a connective tissue disease in which vascular injury is common. The hallmark of SLE, however, are the gross and varied immunological abnormalities (Schur 1985). Autoantibodies figure prominently and have recently been employed in a clinical setting to determine the likely course of the disease. For example anti-DNA antibodies are commonly associated with nephritis and a poor prognosis whilst anti-uRNP antibodies present with patients having Raynauds Phenomenon and a milder course (Schur 1985).

Much of the vascular damage in SLE is thought to result from immune complex (IC) deposition (Miescher et al 1976). The formation of IC by union of antigen and antibody represents a normal physiological process designed to eliminate microbial antigens (Lakha 1978). The presence of persistent or self-perpetuating antigens can lead to an elevation of circulating immune complexes. Large complexes are cleared by the kupffer cells of the liver whilst smaller ones are excreted by the kidneys (Lambert and Casali 1978). Intermediate size complexes tend to localize in the glomerulus and vascular endothelium (Kuffler et al 1987). Here, complexes involving IgM and IgG antibodies activate the classical complement pathway by binding the humoral receptor CI (Lloyd and Schur 1981). Complement activation leads to the generation of C5a and C3b (Hugli and Muller-Eberhaard 1978). C5a, a potent chemotactic agent enhances the accumulation of neutrophils to the site of IC deposition whilst C3b which remains associated with the IC binds to and fixes neutrophils via specific surface receptors. Surface binding of particulate IC to neutrophils induces a profound change in the metabolic activity of

these cells and the generation of cytotoxic reduced oxygen species. Bound IC are also able to stimulate the active extrusion of lysosomal enzymes (Turner et al 1977). In an elegant series of experiments Weissmann has produced evidence to suggest that hydrophobic sites on the Fc portion of aggregated immunoglobin are the sites of engagment with the cell membrane which result in neutrophil activation (Weissman 1979). The importance of neutrophils as mediators of vascular injury has been confirmed in experiment models of IC disease (eg Arthus phenomenon). Animals depleted of neutrophils still form IC on exposure to antigens but not the associated inflammatory response (McClusky et al 1978).

CHAPTER 2

Materials and Methods

Equipment	Supplier
MSE Mistral centrifuge.	MSE Scientific Instruments, Sussex, Great Britain.
Labalux 12 Microscope.	E Leitz LTD, Luton, Great Britain.
Dynatech Automated Micro ELISA Reader.	Dynatech Instruments, California, USA.
Packard Tri-Carb scintillation counter 3255.	United Technologies, Parkard, Berkshire, Great Britain.
LKB UVicord 8300 uv monitor	LKB Uppsala, Sweden.

Materials

All culture reagents and disposable tissue culture plastics.	Gibco Bio-cult LTD Paisley, Scotland.
All fine reagents except where stated otherwise.	Sigma Chemical Company, Poole, Dorset, England.
Percoll.	Pharmacia. Milton Keynes, Bucks, England.
Optiphase Safe Liquid Scintillation Fluid.	LKB, Loughborough, England.
ZK 36374 (Iloprost).	Scheering AG, Cologne, West Germany.
^{14}C Arachidonic Acid (50 u Ci/ml).	Amersham, Aylesbury, Bucks.
Factor VIII Rag rabbit anti-human antiserum and Factor VIII Rag peroxidase congugate	Dako LTD, High Wycomb, Bucks.

Cell Separation Reagents

1. Dextran Solution

Dextran (M wt 500,000)		6 g
PBSG	to	100 mls

2. Percoll Solution (iso-osmotic)

Stock percoll	90 ml
PBS (10 x strength)	10 ml

3. Percoll Solution (1.083 g/l)

Iso-osmotic percoll	65 ml
PBSG (single strength)	35 ml

Adjusted to 1.083 g/l with hydrometer

4. Red Cell Lysis Buffer

Ammonium chloride		0.83 g
Distilled water	to	100 ml

5. Phosphate Buffered Saline + glucose (PBSG)

PBS tablets (oxoid)		10
Glucose		1.6 g
Distilled H_2O	to	1 l

Reagents for Superoxide/Hydrogen Peroxide Assay

1. **Phenol Red Buffer Supplemented with Horseradish Peroxidase (PRSB - HRPO)**

Phenol red		0.020 g
Horseradish peroxidase (200 u/mg type 2)		0.0056 g
PBSG	to	100 ml

2. **Cytochrome C Buffer**

Cytochrome C (horse heart type 3)		0.197 g
PBSG	to	100 ml

Myeloperoxidase Assay

1. **Citric Acid Buffer (0.1M)**

Citric acid		2.101 g
Distilled water	to	100 mls

2. **Phosphate Buffer**

Sodium biphosphate (Na_2 HPO_4 $12H_2O$)		3.58 g
Distilled water	to	100 mls

3. **Citrate Phosphate Buffer**

Citric acid buffer	50 mls
Phosphate buffer	50 mls
pH to 5.5 by titration then supplement with	
triton x:100	0.05%
Hydrogen peroxide (30%)	10 ul

4. **MPO Reaction Buffer**

IM. HCL	1ml
Ethanol (90%)	9 ml
O-dianisidine	0.244 g
Dilute in citrate phosphate buffer to	3.2%

Reagents for Chemotaxis Assays

1. Hanks' Ballanced Salt Solution Normal x Strength

HBSS (10 x strength)		10.0 mls
Sodium bicarbonate (7.5% w/v)		2.7 mls
Penicillin (5000 u/ml)		1.0 ml
Streptomycin (5mg/ml)		1.0 ml
Distiled water	to	100 mls

pH 7.3

2. HBSS Supplemented with MOPS (HBSS/MOPS)

MOPS		0.201 g
HBSS to		100 mls

3. Minimum Essential Media (MEM 2 x strength)

MEM (10 x strength)		20 mls
Foetal calf serum		20 mls
Penicillin (3000 u/ml)		2.0 mls
Streptomycin (5 mg/ml)		2.0 mls
Sodium Biocarbonate		5.4 mls
Distilled water	to	100 mls

4. **Agarose Gel**

Agarose	2.0 g
Distilled water (100°C) to	100 mls
Cool to 65°C	
Mix with 2 x MEM (65°C)	100 ml

Reagents for F VIII Rag assay

1. **Substrate**

1, 2 ortho phenylenediamine dylhydrochloride.		0.008 g
Citrate phosphate buffer	to	15 mls
Supplement with H_2O_2 (20 vol)		10 ul

2. **Carbonate Buffer** (0.05 mol/l

$Na_2\ CO_3$		1.59 g
$NaH\ CO_3$		2.93 g
NaN_3		0.2 g
Distilled water	to	1000 ml
pH 9.6		

3. **0.5 ml/1 PBS Tween**

Tween 20		0.5 ml
PBS	to	1000 ml

4. **Citrate Phosphate Buffer** (0.1 mol/L)

$C_3H_4OH\ (COOH)_3$		7.3 g
$Na_2\ HPO_4\ 12H_2O$		23.87 g
Distilled water	to	1000 mls

Methods

Source of Experimental Blood and Serum

Drug and inhibitor studies (with the exception of the iloprost trial) were carried out on neutrophils from normal healthy volunteers.

In the patient studies blood was obtained with informed consent from patients with the help of Dr N McHugh and Dr P J Maddison. Frequently subjects were obtained at the beginning of parallel running clinical trials and were not receiving any drug treatments. Where possible patient studies were accompanied by age and sex matched controls. With respect to the patient diagnosis H_2O_2 assays were carried out blind.

Serum samples were obtained for normal healthy individuals from a blood donor clinic. Patient samples were obtained retrospectively from a central store and where possible at dates closer to when the neutrophil studies were undertaken.

Preparation of Stimuli

Aggregated IgG

IgG was prepared by affinity chromatography by passing diluted human myeloma serum down a protein A column. The IgG in the serum binds to protein A through its Fc receptor in a pH dependent fashion. Once bound the IgG is eluted by lowering the pH of the column.

Sera obtained from an IgG myeloma patient was centrifuged at 2000g to remove particulate matter and then diluted to 50% with PBS. Aliquots of 4 ms of serum wre then added to a protein A column in a step wise fashion. The column was then washed with 10/150 phosphate buffer to remove unwanted contaminants. Acetic acid (1 mol/1) was then added to the top of the column and bound IgG eluted into a small beaker. The preparation was then left to dialyse in PBS at 4°C (with frequent changes of buffer) until the pH was neutral. The protein concentration of the sample was determined spectrophotometrically and the sample concentrated as required and stored at -20°C.

Aggregated IgG was prepared by thrawing an aliquot of the concetrate and heating at 63°C for 30 minutes

FMLP and PMA

FMLP and PMA were diluted in DMSO and subsequently in PBSG to the desired concentration. The DMSO concentration in the final assays was never allowed to exceed 0.2%. This concentration had no antioxidant activity against superoxide generated by a xanthine/xanthine oxidase system or against the phenol red oxidation produced by 50 uM H_2O_2.

Neutrophil Purification

Neutrophils were purified by density gradient centrifugation of erythrocyte depleted whole blood, using a modified method of Segal et al (1980)

Heparinised venous blood (4 u/ml preservative free heparin) was diluted by 50% with PBSG). 8 mls of diluted blood were then thoroughly mixed with 2 mls dextran (m wt 500,000 diluted to 6% w/v with PBSG) in polycarbonate test tubes (to prevent adherence) and allowed to stand for 30 minutes at room temperature, after which time the majority of erythrocytes had sedimented to the bottom of the tube. The leukocyte rich suspension was then carefully layered onto 2 mls of Percoll (1.083 g/l) and Methods) and centrifuged for 30 mins at 400 g at room temperature.

The centriguation process separated two cell populations. Mononuclear cells remained in a band at the interphase between the Percoll and plasma whilst neutrophils passed through the Percoll layer to form a pellet together with any residual erythrocytes. The cell pellet was carefully isolated, transferred to a new polycarbonate centrifuge tube and washed once in PBSG. After washing, the PBSG was removed and the cell pellet was resuspended in 2 mls of sterile ammonium chloride (0.83% w/v in distilled water) to lyse the remaining red cells. The disrupted cell suspension pellet was left in contact with ammonium chloride for 10 minutes at 37°C and then neutralized by adding 10 mls of PBSG.

After a further two washes by centrifugation in PBSG at 150 g the cells were resuspended in 1 ml of appropriate medium and counted by microscopy in white cell counting fluid. Cells prepared in this way were consistently

> 95% polymorphonuclear leukocytes as assessed by morphology (ie neutrophils) and > 95% viable as assessed by trypan blue exclusion.

Hydrogen Peroxide Assay

The measurement of hydrogen peroxide release from cultured cells is based on the method described by Pick & Keisari (1980, 1981). In this assay hydrogen peroxide oxidises phenol red in the presence of the enzyme horseradish peroxidase. The oxidised product exhibits an increased absorbance at 610 under alkaline conditions (pH 12.5) and so can be monitored spectrophotometrically. The change in absorbance is directly related to the H_2O_2 concentration and absorbance values can be converted to H_2O_2 concentrations using a previously constructed standard curve.

Construction of Standard Curve

To a 100 ul of hydrogen peroxide solution at the desired concentration were added to 200 ul of PBSG and 700 ul of phenol red buffer supplemented with HRPO (PRB-HRPO) (11.4 u/ml). The mixture was then incubated for 30 minutes at 37°C and the reaction terminated by cooling on ice. Aliquots of 10 ul of 3 M NaOH were then added to each tube and mixed and 200 ul of each sample transferred to a microtitre plate and analysed spectrophotometrically on an ELISA reader at 610 nm. Absorbance values for each concentration of H_2O_2 were adjusted by subtracting blank values (as sample but without H_2O_2) and plotted against H_2O_2 concentration to construct a standard curve.

Neutrophil Derived H_2O_2

Conditions were similar to those used for the construction of the standard curve except reagent H_2O_2 was replaced by 100 ul of pure neutrophils (10^6 cells). These were added to 700 ul of HRPO-PRS, 100 ul of PBSG and 100 ul of stimulant or buffer. Following a 30 minute incubation period at 37°C the reaction was stopped by cooling rapidly to 4°C and the neutrophils removed by centrifugation. To the cell-free supernantants 10 ul of 3M NOAH were added. Aliquots of 200 ul were then analysed spectrophotometrically. Each incubation was performed in duplicate.

Inhibitor Studies

Inhibitors were either preincubated with cells for the appropriate time periods or added direct to the assay mixture. Where preincubation was required and cells were then added to the assay mixture, any 'washout phenomenon' was assessed by including or excluding the appropriate concentration of inhibitor in the reaction buffer. A fall in inhibition in the presence of buffer indicated washout phenomenon and reversibility of inhibition. Where this occurred the inhibitor was also added to the assay mixture.

Superoxide Anion Assay

Measurment of superoxide was carried out using a modified method of Pick and Mizel (1981). Superoxide generated from cellular and enzyme sources was measured by its ability to reduce cytochrome C This can be monitored spectrophotometrically at 550 nm. Neutrophils were suspended at a concentration of 1 x 10^6 cells/ml in 160 uM cytochrome C and incubated with buffer or stimulants for 15 minutes at 37°C. The reaction was then stopped by reducing the temperature to 4°C by placing the reaction tubes in iced water. The cells were then centrifuged at 150 g at 4°C and 200 ul aliquots of the supernatant were added to microwell plates. Absorbance values were determined on an automated ELISA reader set to 550 nm. The absorbance expressed was calculated as the difference between that produced by cells in the absence of superoxide dismutase (100 u/ml) and that produced in its presence to give an increse in absorbance specific to superoxide.

Results are expressed in terms of n moles of cytochrome C reduced by 10^6 cells in 15 minutes as determined from the following formula:

$$A = Ecl$$

Where A = absorbance

E = extinction coefficient of cytochrome C

c = concentration of cytochrome C

l = length of light path

Neutrophil Polarisation Assay

Neutrophil polarisation was measured according to the method of Haston and Shields (1984, 1985). In response to a uniform concentration of a chemotactic agent, human neutrophils acquire a polarised morphology. The degree of polarity and the number of cells polarised is dependent upon the concentration of chemotactic agent (Haston and Shields 1984).

Cell Preparation for Polarisation Assay

Purified neutrophils were suspended in HBSS supplemented with MOPS at a concentration of 1 x 10^6 cells/ml. They were then incubated with chemotactic agents for 30 minutes at 37°C. Following this cells were then fixed by adding 2 mls of 2.5 v/v glutaralderyde in HBSS/MOPS for 10 minutes and finally washed twice by centrifugation at 150 g in HBSS/MOPS. The fixed cells were then resuspended in a drop of medium and examined under phase contrast microscopy. Any cell deviating from a spherical outline was counted as polarised. Three groups each of 100 cells were examined in a 'blind' fashion for changes in polarity.

Neutrophil Chemotaxis Assay

Neutrophil chemotaxis was measured under agarose according to the method of Nelson et al (1975). The technique has several advantages over traditional chemotaxis assays (Boyden 1953) in that it is cheap and simple to set up, requires fewer cells, and measures both spontaneous and chemotactic migration simultaneously.

Preparation of Agarose Plates

Agarose (A 6013, Sigma) was dissolved in boiling sterile distilled water at a concentration of 2% w/v. After cooling to 65°C it was mixed with an equal volume of prewarmed (65°C) 2 x concentration minimum essential medium (Eagles) containing 20% v/v foetal calf serum, glutamine and antibiotics. Aliquots of 8 mls of the mixture were then transferred to 2.5 cm diameter sterile petri dishes and allowed to set.

Six series of three wells 2.4 mm apart were cut in the agarose using a template and cutting tool. For each group of three wells chemotactic agent, cells and buffer were added to the outer, central and inner wells respectively and allowed to incubate for 3 hrs at 37°C humidified chamber. Following incubation the cells were fixed by the addition of methanol (3 mls/dish) for 30 minutes and then formaldehyde (3 mls/dish) for a further 30 minutes. The agarose gel was then removed and the fixed cells stained for 15 minutes with Wrights stain.

Cell movement was assessed using a projecting microscope and the chemotactic index (CI) calculated from the formula below.

$$CI = \frac{\text{DISTANCE MOVED TOWARD CF}}{\text{DISTANCE MOVED TOWARDS BUFFER}} - \frac{\text{CHEMOTACTIC MIGRATION}}{\text{RANDOM MIGRATION}}$$

Determinations for each experiment were carried out in sextruplicate and on three separate agorose plates (18 values in all). The effect of inhibitors were assessed by preincubating the cells with inhibitor for the indicated times prior to addition to the wells.

Myeloperoxidase Assay

Myeloperoxidase was assayed according to the method of Segal et al (1980). Purified neutrophils (5 x 10^6/ml) were incubated with either buffer (to measure basal enzyme release) or aggregated IgG (200 ug/ml) or triton X-100 (0.2%) (to measure total enzyme release) for 30 minutes at 37°C. Subsequent to this the cell suspensions were centrifuged at 2000 g for 15 minutes (4°C). 50 ul of supernatant were then added to the 3.2 ml of the MPO reaction buffer (see Materials and Methods) and incubated for 5 minutes at 37°C. After this time 1 ml of perchloric acid was added to each sample and the colour reaction measured on a spectrophotometer set to 560 nm. Determinations of basal and stimulated MPO activity were carried out in duplicate and all values were expressed as the percent of total cellular myeloperoxidase (ie MPO release caused by triton X-100 mediated cell lysis).

Factor VIII Related Antigen Assay

Factor VIII related antigen (FVIII Rag) forms a large part of the circulating factor VIII complex which consists of factor VIIIc (a clotting factor) and von Willebrand factor (a platelet adhesion factor) (Kahaleh et al 1981). Endothelial cells are responsible for the synthesis of FVIII Rag and it is now recognised that physical or chemical trauma to then increase levels of FVIII Rag in the circulation (Lee et al 1985). Vasculitis is a clinicopathological process characterised by inflammation and necrosis of the blood vessels and elevated serum levels of FVII Rag (Belch et al 1986). In view of this, FVIII Rag levels were measured in connective tissue disease patients where vascular injury is common to the pathology. Sera from

patients with SLE, MCTD, SS and PR were obtained from a serum bank (maintained at - 70°C) and diluted as required in PBS supplemented with 1 ml/litre TWEEN. Aliquots of 100 ul of sera were then added in duplicate to microwell plates which had been coated with FVIII Rag antiserum (100 ul of antiserum diluted 1/1000 in carbonate buffer and incubated overnight at 4°C and then washed in 3 titres in PBS supplemented with 0.5 ml/litre TWEEN. Following a 1 hr incubation in a humidified incubator at 37°C the sera was removed by agitation and the microwell plates washed as before. After removing excess buffer 100 ul of anti-human factor VIII Rag - peroxidase conjugate diluted 1/1000 were added to each well and left to incubate for 1 hr at 37°C. The washing procedure was then repeated as before with an extra wash in citrate buffer. After a further drying step 100 ul of substrate (orthophenylene diamine dihydrochloride) supplemented with H_2O_2 (10 ul of 20 volumes) were added to each microwell and left to develop.

Construction of a Standard Curve

A standard curve was prepared by adding diluted pooled normal human serum to the microwells (1/10 - 1/320 dilutions) and following the proceedure described previously. A new standard curve was constructed for each experiment and a 1/10 standard serum dilutation was added to each microwell plate so that colour development on different microwell plates could be monitored in synchronization.

Colour development was measured on an automated elisa reader (transmission 490 nm) when the 1/10 standard serum dilution attained an

optical density of 1.1. Aliquots of 100 ul of PBS supplemented with TWEEN were added to 6 microwells on each plate to act as blanks.

Interpretation of Results

The average readings from the blanks were substrated from all other readings. These were then read off the standard curve and multiplied by the dilution to give a value for F VIII Rag levels expressed as a percent of that in the pooled normal standard serum.

Statistical Analysis of Data

Statistical significances in the pharmacological studies employing neutrophils from healthy subjects were tested for using the paired students T-test.

Comparison of data between different patient/control groups was achieved using the Mann-Whitney U test for non-parametric data. Correlation coefficient between different sets of data were obtained from the Spearmans rank correlation coefficient for non parametric data. All statistical methods were performed using the statistics package (Interstat) supplied with the Apple IIE Computer (editied by Bruce Land 1979).

CHAPTER 3

Studies on the Origin of Stimulated and Basal H_2O_2 Production in Human Neutrophils

Results

Neutrophil activation, following exposure to particulate matter or inflammatory mediators is often accompanied by a marked increase in oxidative metabolism and the generation of $O_2^{\cdot}$ and H_2O_2 (Goldstein et al 1975, Simchowitz and Spillberg 1979, Simchowitz et al 1982). That these reactive oxygen species can be detected in the medium surrounding stimulated cells has important biological implications in view of their potential reactivity with organic molecules (Joke et al 1975, Henson and Johnston 1987). In this thesis H_2O_2 has been measured as an index of neutrophil oxidative metabolism. Unlike $O_2^{\cdot}$, H_2O_2 can freely diffuse through cell and organelle membranes (Ohno and Gallin 1985) and therefore basal levels can be detected in unstimulated (resting) cells. Superoxide on the other hand is rapidly bound and degraded by intracellular dismutase enzymes so that only negligible levels can be measured in resting cells (Fridovich 1978).

In the final chapter of this results section, H_2O_2 production has been measured in neutrophils from a variety of connective tissue diseases in which microvascular damage forms an important component. The aim of this section was to determine whether neutrophil derived oxidants could be contributing to the microvascular injury. With reference to this, H_2O_2 but not $O_2^{\cdot}$, has been shown to exhibit direct cytotoxic activity and

therefore in this context measurement of H_2O_2 would be the most appropriate indicator of the cytotoxic potential of these cells (Ager and Gordon 1984).

Measurement of cell derived H_2O_2

The H_2O_2 dependent, horseradish peroxidase mediated oxidation of phenol red was employed to assay cell derived H_2O_2 (Pick and Keisari 1980). Oxidised phenol red exhibits an increased absorbance (610 nm) at alkaline pH which can be measured spectrophotometrically. Absolute H_2O_2 production was determined from a previously constructed standard curve (Fig 3). Between 0-50 uM H_2O_2 a linear relationship between increased phenol red absorbance and H_2O_2 concentration was observed. This was subsequently found to encompass the full range of stimulated H_2O_2 responses.

Prior to any inhibitor or patient studies concentration response curves were constructed for each stimulus to be employed. The 30 minute incubation/reaction time with stimulants follows those established by other workers (Pick and Mizel 1981, Maslen 1986). All stimulants employed generated H_2O_2 in a concentration dependent manner, but maximal H_2O_2 generation varied with each stimulant (Figs 4-6). Basal (unstimulatd) H_2O_2 generation was also measured over a 30 minute period by incubating

cells with buffer (PBSG) instead of the stimulus. This varied considerably between individuals with a mean value of 2.4 ± 1.9 uM $H_2O_2/10^6$ cells/30 minutes.

Table 2 summarises the data obtained from Figures 4-6.

Figure 3 - CURVE FOR STANDARDISING THE PHENOL RED ASSAY.

The effect of reagent H_2O_2 on phenol red absorbance (610 nm).

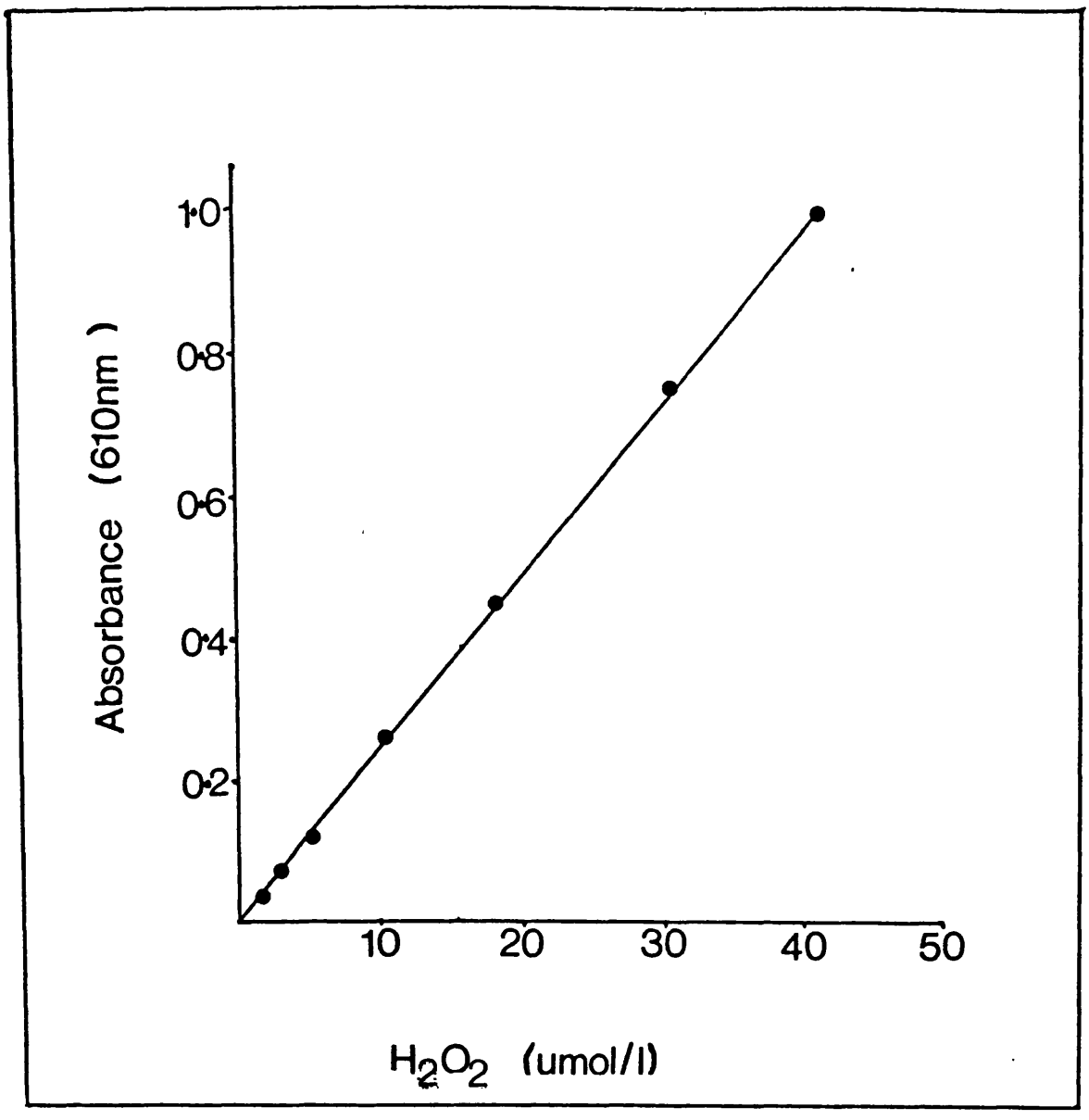


Figure 4 - NEUTROPHIL H_2O_2 PRODUCTION STIMULATED BY FMLP

Human neutrophils were incubated in the H_2O_2 assay mixture containing FMLP at various concentrations or buffer, to measure background H_2O_2 production. H_2O_2 response is expressed in umol/l /1 x 10^6 cells/30 minutes.

Points represent the mean ± standard deviation of 4 experiments (all values corrected for background H_2O_2).

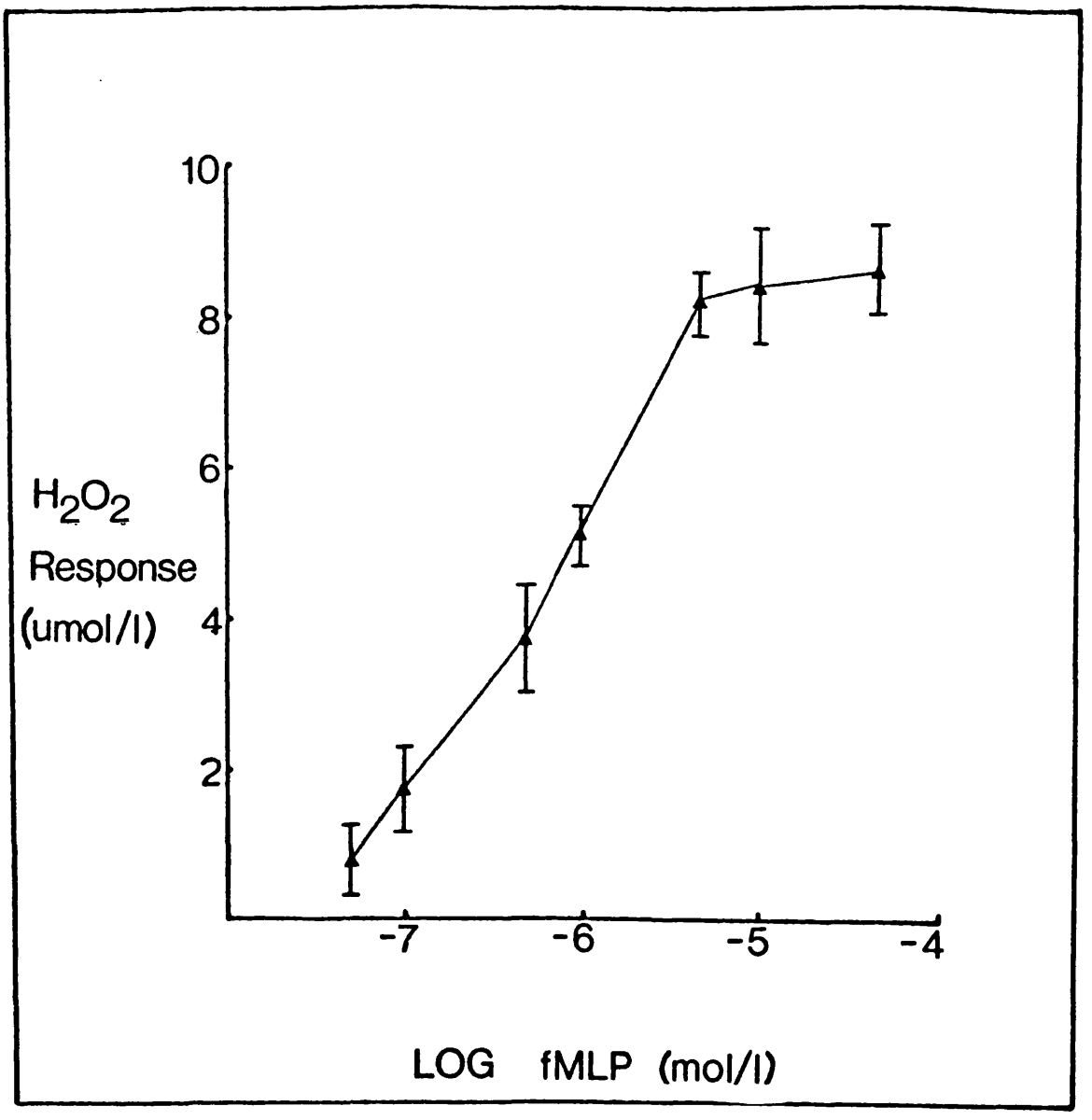


Figure 5 - NEUTROPHIL H_2O_2 PRODUCTION STIMULATED BY AGGREGATED IgG

Human neutrophils were incubated in the H_2O_2 assay mixture containing aggregated IgG at various concentrations or buffer to measure background H_2O_2 production. H_2O_2 response is expressed in umol/1 /1 x 10^6 cells/30 minutes.

Points represent mean ± standard deviation of 4 experiments (all values corrected for background H_2O_2 production)

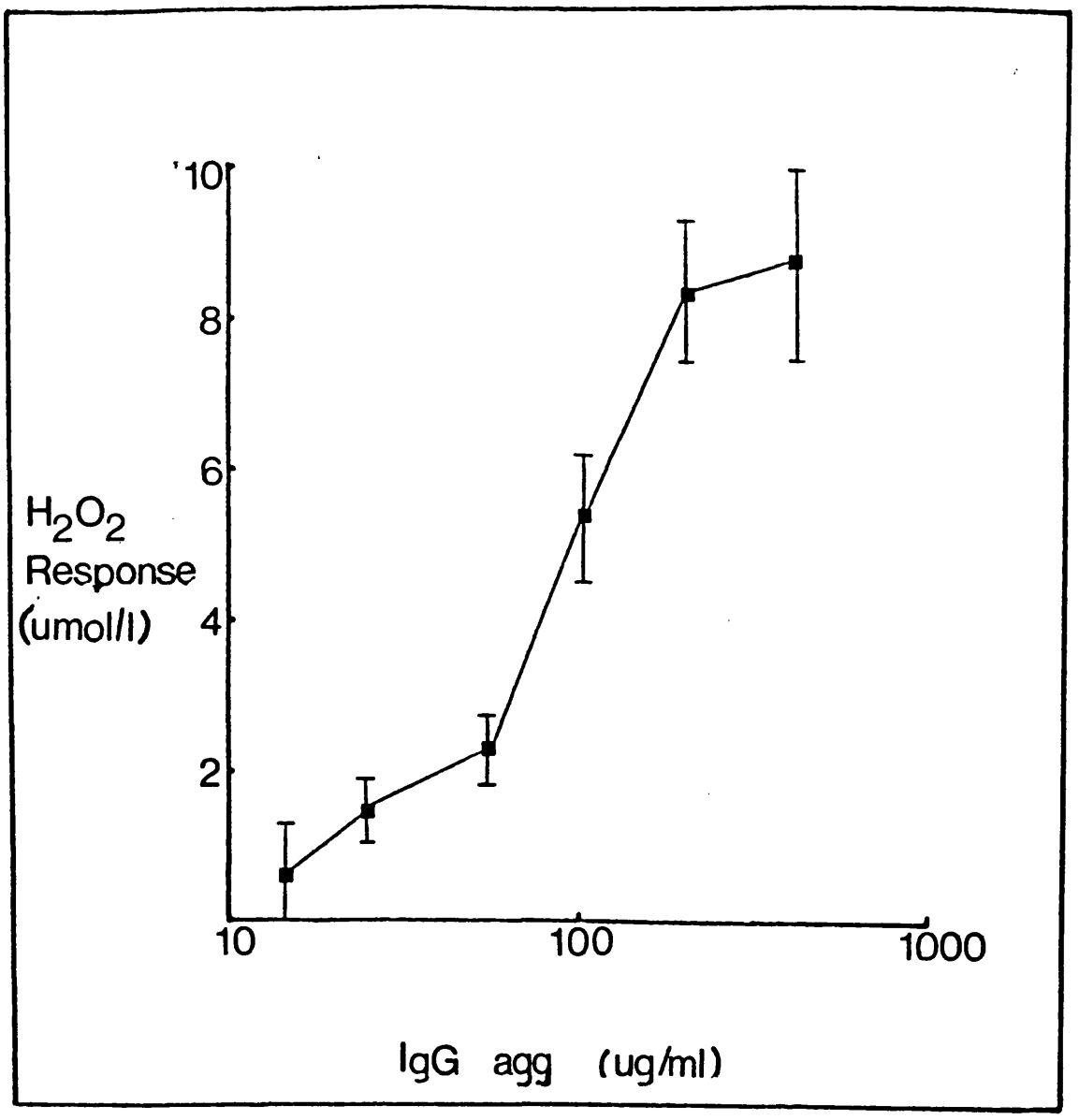


Figure 6 - NEUTROPHIL H_2O_2 PRODUCTION STIMULATED BY PHORBOL MYRISTATE ACETATE (PMA)

Human neutrophils were incubated in the H_2O_2 assay mixture containing PMA at various concentrations or buffer to measure background H_2O_2 production. H_2O_2 response is expressed in umol/l /1 x 10^6 cells/30 minutes.

Points represent the mean ± standard deviation of 4 experiments (all values corrected for background H_2O_2 production).

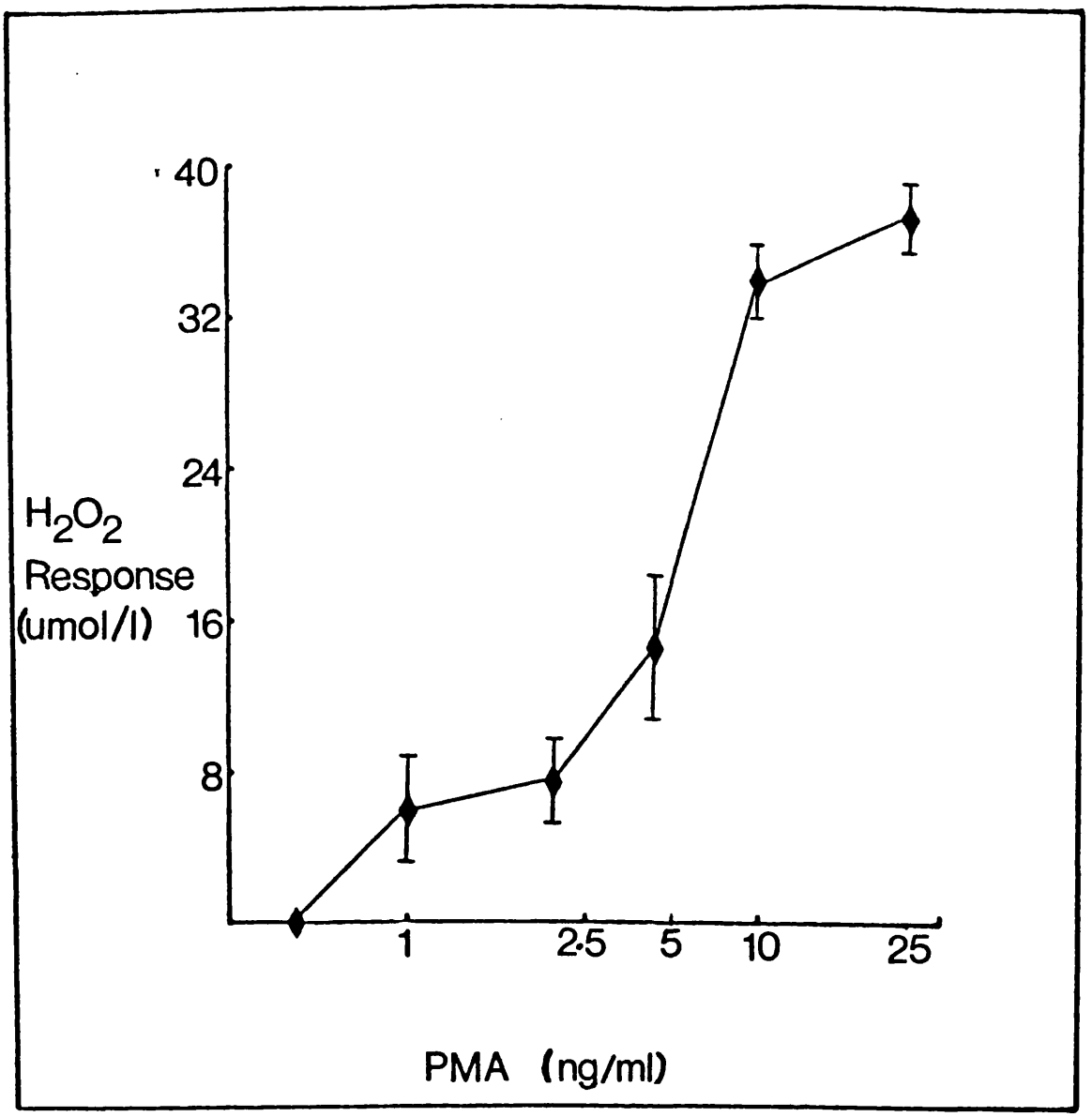


Table 2

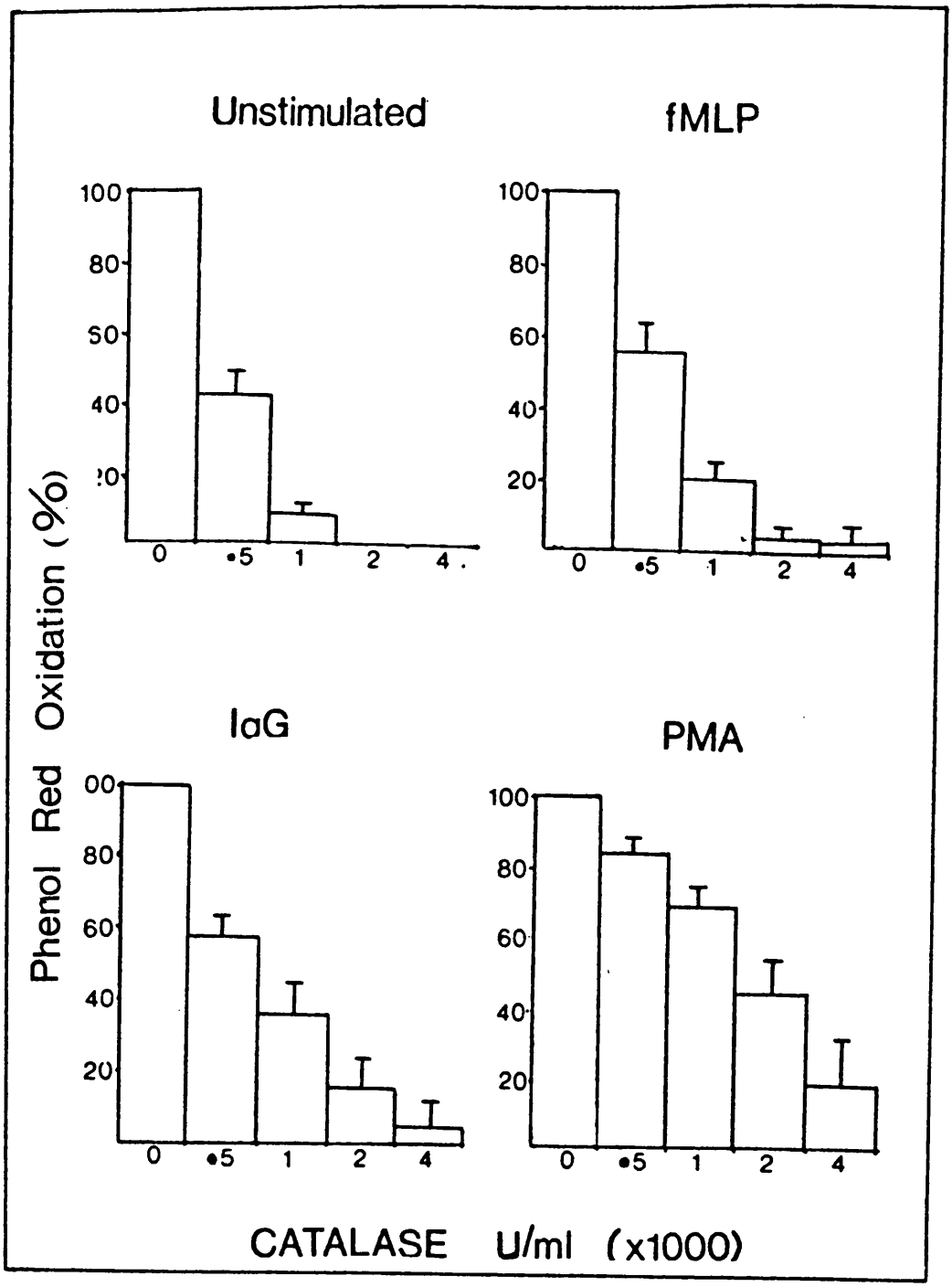
Resting and stimulated H_2O_2 responses expressed as ED50 values (ie the concentration of stimulant required to generate a 50% maximal response) and absolute maximum response (mean ± SD).

STIMULUS	ED50	MAXIMUM RESPONSE
		uM H_2O_2
BUFFER		2.4 ± 1.9
FMLP	6.5×10^{-7} M	9.1 ± 0.8
IgG (AGG)	85 ug/ml	9.6 ± 1.4
FMLP + IGG (AGG)		21.4 ± 3.6
PMA	7.4 ng/ml	37.5 ± 2.0

To determine the specificity of the assay for hydrogen peroxide, the effects of various concentrations of catalase on resting and stimulated phenol red oxidation were assessed (Fig 7). Unstimulated and FMLP stimulated phenol red oxidation were very sensitive to the effects of catalase, being abolished at under 2000 u/ml. The IgG and PMA responses were less sensitive to the effects of catalase. At 4000 u/ml of catalase the IgG stimulated phenol red oxidation was almost completely abolished. However, even at this concentration only 80% of the response to PMA was inhibited. The effects of catalase at these high concentrations still appeared to be enzyme specific because the addition of an equivalent concentration of protein (bovine serum albumin) had minimal effects upon stimulated phenol red oxidation.

Figure 7 - THE EFFECTS OF CATALASE ON BASAL AND STMULATED PHENOL RED OXIDATION

Human neutrophils were incubated in the H_2O_2 assay mixture containing buffer, FMLP, (5 x 10^{-6}M) aggregated IgG (200 ug/ml), or PMA (10 ng/ml) with, or without catalase. Results are expressed as a percent of the phenol red oxidation that occurred with each stimulant in the absence of catalase.



The Origin of Cell derived H_2O

The origin of cell derived H_2O_2 was investigated using diphenylene iodonium (DPI - a generous gift from Prof O T G Jones - University of Bristol). DPI has been reported to inhibit neutrophil superoxide production in response to PMA by binding to a 45kDa component of NADPH oxidase (Cross and Jones 1986). Initially, the effects of DPI on FMLP stimulated $O_2^{\cdot}$ and H_2O_2 were compared in order to ascertain whether both species were derived from the same DPI-sensitive enzyme (ie NADPH oxidase) (Fig 8). The effects of DPI were extremely rapid, full inhibition being achieved within the mixing time. Furthermore, the inhibition of FMLP stimulated oxidative responses was not affected by washout (ie was apparently irreversible). Similar inhibition profiles were obtained for both superoxide and hydrogen peroxide production indicating that both originated from the same enzyme. The effects of DPI on H_2O_2 production in response to FMLP, IgG (aggregated) and PMA and in the absence of a stimulant are shown in Fig 9. Similar inhibition profiles were observed for both unstimulated and stimulated responses as would be expected if they were all generating H_2O_2 through NADPH oxidase.

Having established the source of cell derived H_2O_2 the factors affecting unstimulated H_2O_2 production were investigated. Addition of calcium ions to the neutrophil suspension enhanced resting H_2O_2 production dose dependently (Fig 10). At 1mM calcium ion concentration (ie a near physiological concentration of extracellular calcium) the basal response was stimulated by some 300%.

Cytochalasin B has been reported to enhance the neutrophil oxidative response stimulated by FMLP (Root and Metcalf 1977). However in this study cytochalasin B inhibited unstimulated H_2O_2 production dose dependently with almost complete inhibition being achieved at 5 ug/ml (fig 11). This concentration of cytochalasin B is regularly employed by other workers to enhance FMLP stimulated oxidative metabolism.

Figure 8 - THE EFFECTS OF DPI ON FMLP STIMULATED SUPEROXIDE AND HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were incubated in the H_2O_2 assay mixture (▲——▲) or $O_2^{\cdot-}$ assay mixture (△——△) containing FMLP (5×10^{-6}M) and DPI at various concentrations. Results are expressed as percent of the oxidative response that occurred with FMLP in the absence of DPI.

Points represent the mean ± standard deviation of 3 experiments.

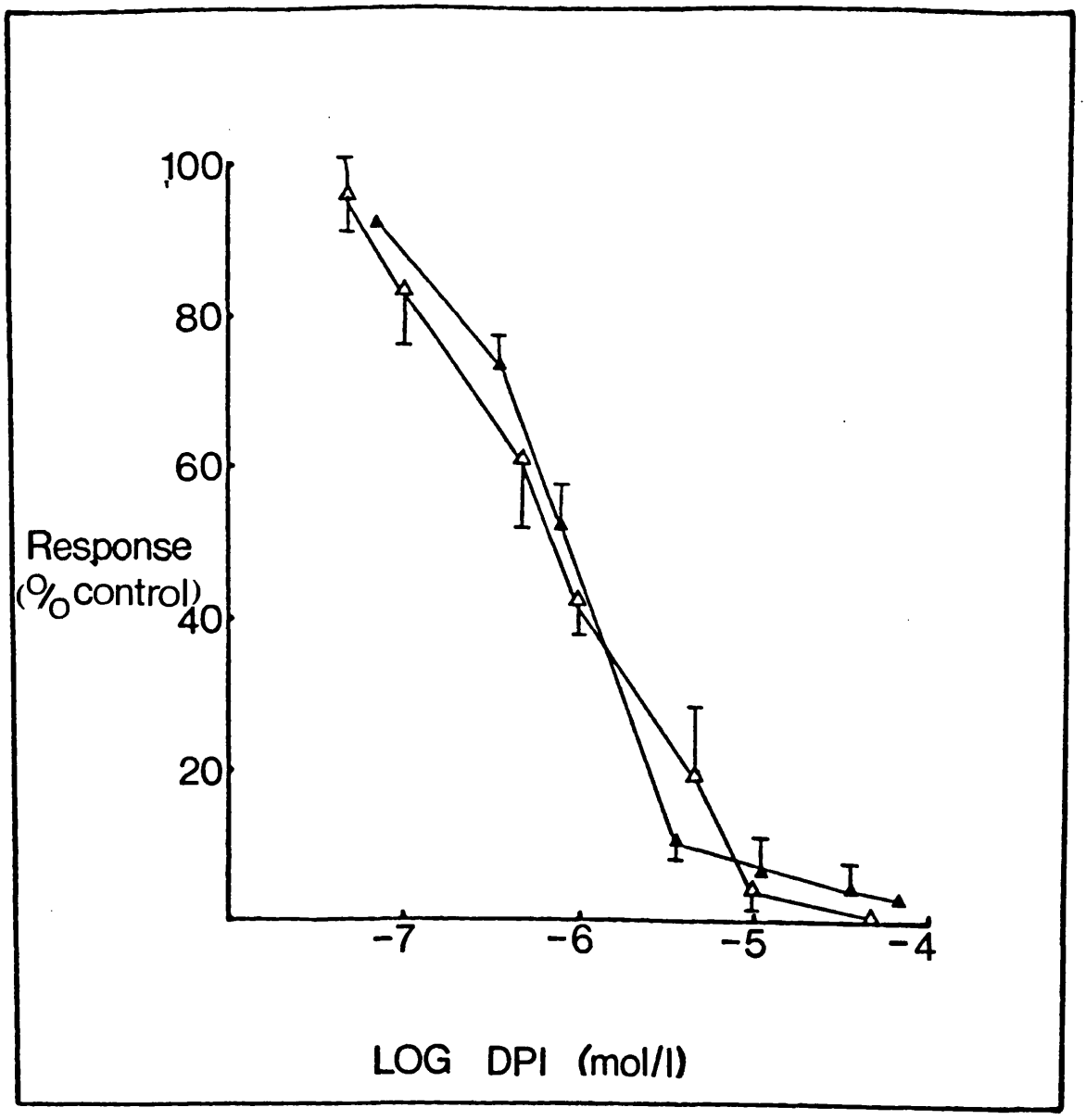


Figure 9 - THE EFFECT OF DPI ON BASAL AND STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were incubated in the H_2O_2 assay mixture containing FMLP (5×10^{-6}M ▲——▲), aggregated IgG (200 ug/ml ■——■), PMA (10 ng/ml ◆——◆) and buffer (●——●) with various concentrations of DPI. Results are expressed as percent of the H_2O_2 produced by the stimulants in the absence of DPI.

Points represent the mean of 4 experiments (standard deviation bars omitted for sake of clarity.)

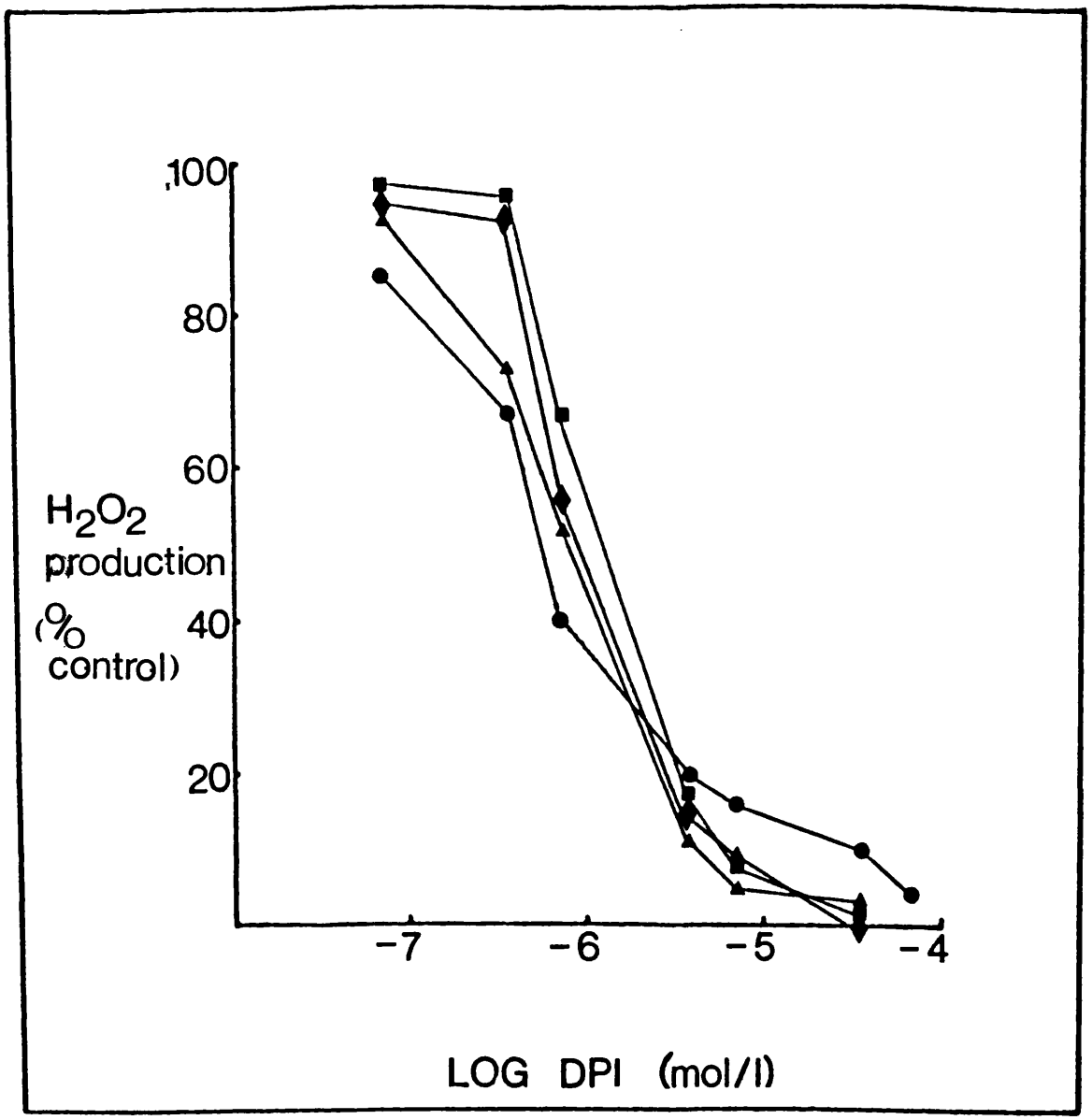


Figure 10 - THE EFFECTS OF CALCIUM IONS ON UNSTIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were incubated with the H_2O_2 assay mixture supplemented with calcium ions at various concentrations. Results are expressed as a percent of the unstimulated H_2O_2 production that occurred in the absence of calcium.

Bars represent the mean values of 2 experiments.

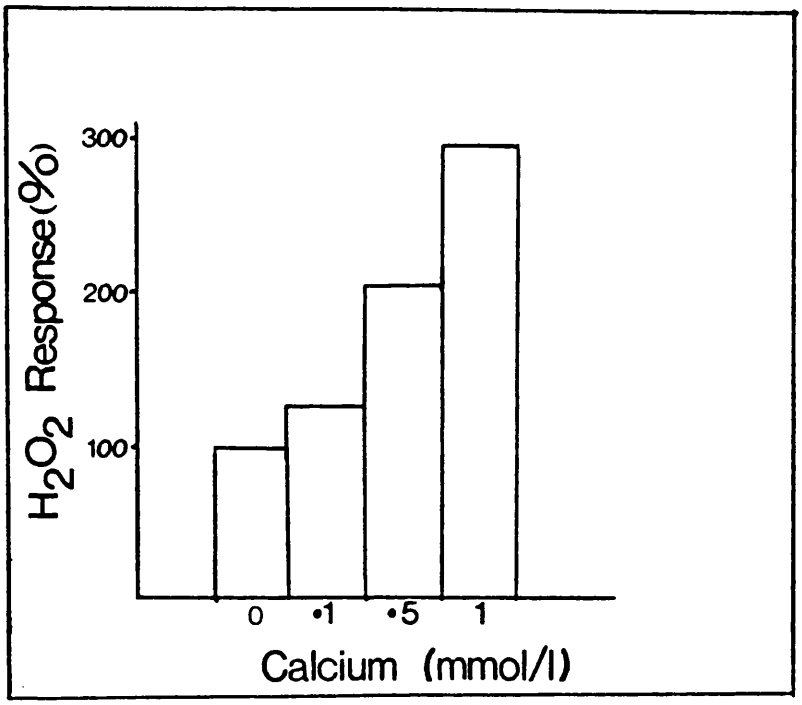
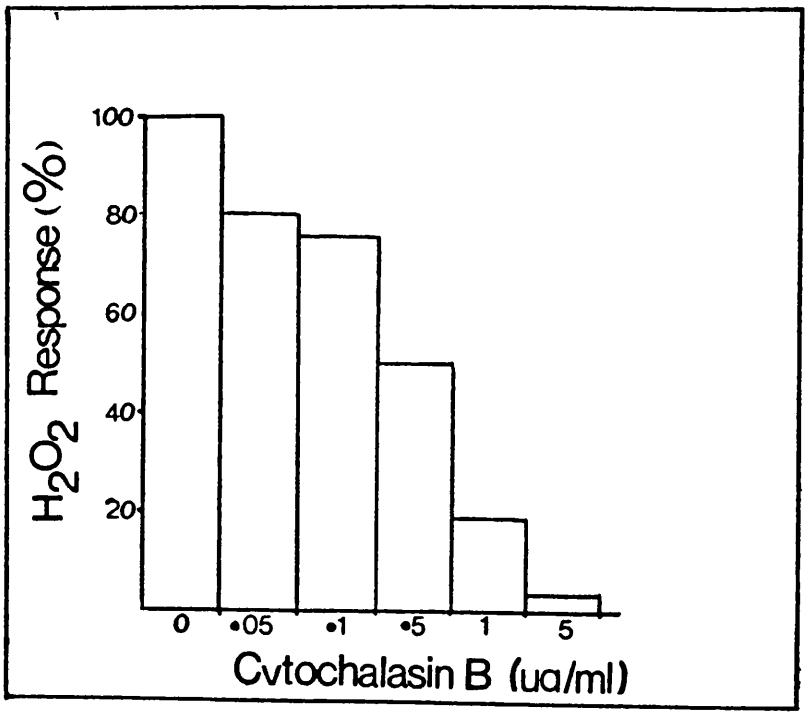


Figure 11 - THE EFFECT OF PREINCUBATNG NEUTROPHILS WITH CYTOCHALASIN B ON UNSTIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were preincubated for five minutes at 37°C with cytochalasin B and then added to the H_2O_2 reaction assay mixture containing buffer. Results are expressed as the percent of the H_2O_2 response that occurred in the absence of cytochalasin B.

Bars represent the mean values of 2 experiments.



CHAPTER 4

The Effects of Agents That Increase Intracellular Cyclic Adenosine Monophosphate Upon Neutrophil Oxidative Metabolism

Agents that elevate intracellular cAMP levels have been reported to inhibit different aspects of neutrophil activity (eg lysosomal enzyme release and phagocytosis) (Ignarro and Cech 1976). In this study the effects of four such compounds, adrenaline, salbutamol, adenosine and the synthetic prostacyclin analogue ZK 36374 (Iloprost) were examined on unstimulated and stimulated neutrophil hydrogen peroxide production in vitro. In addition, the effects of intravenous Iloprost infusion in patients with systemic sclerosis, upon subsequently stimulated neutrophil oxidative respones in vitro were investigated.

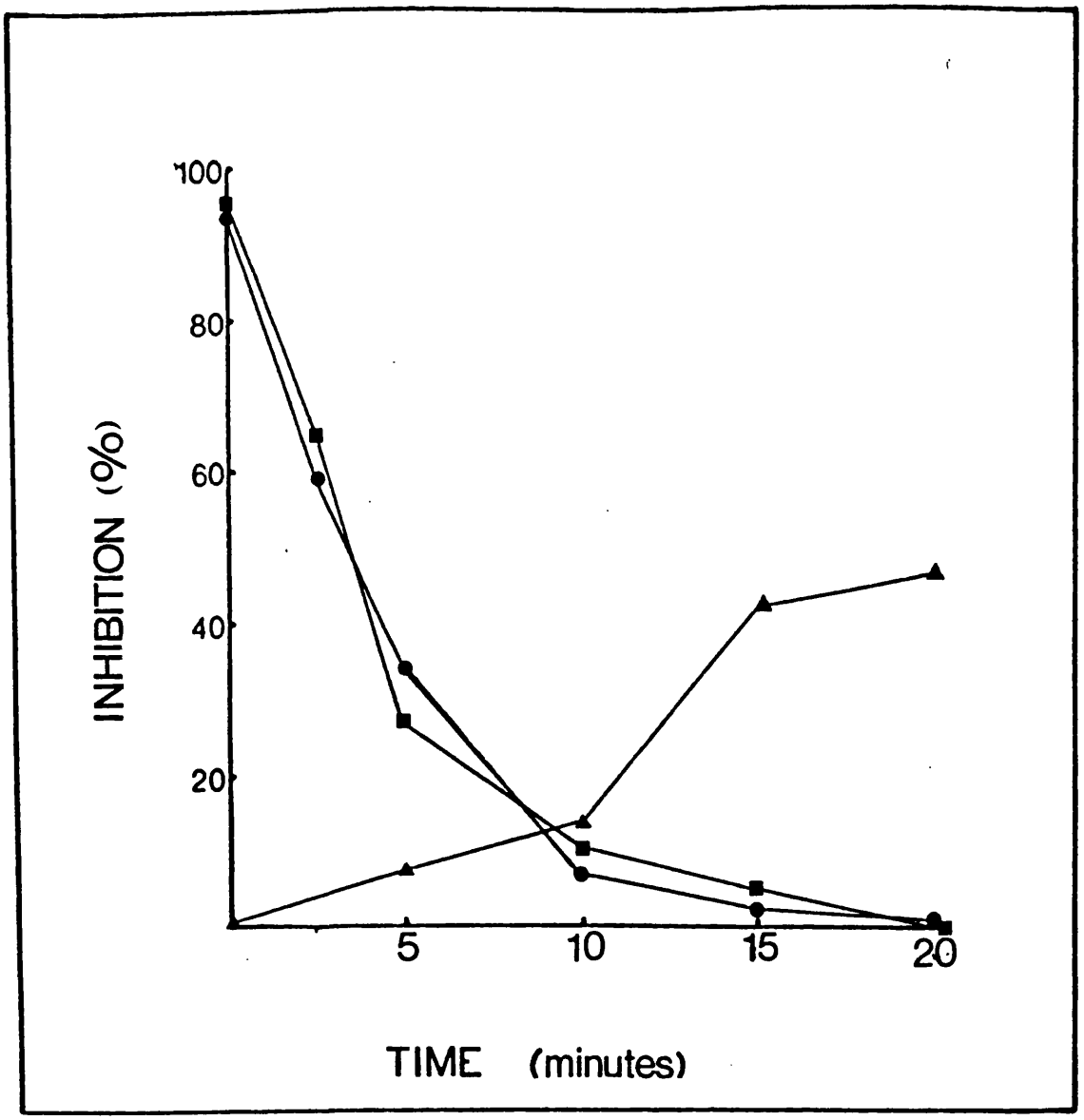
Time course studies were undertaken to determine the preincubation time periods required for optimal inhibition by each agent. Neutrophils were preincubated with each agent for the indicated time period and then added to the reaction mixture containing stimulant. Where preincubation times are indicated as zero, cells were added directly to the reaction mixture containing both stimulant and inhibitor. The concentrations of inhibitors employed for time course studies were those reported as being effective in the literature (Ignarro et al 1974, Cronstein et al 1983). Fig 12 illustrates the results of these time course studies utilising FMLP as the stimulant. Both adrenaline (5×10^{-5}M) and Iloprost (1.4×10^{-4}M) produced optimal inhibition when added to the cells in the presence of FMLP: ie without preincubation. With increased pre-incubation times, inhibition by these agents was reduced. Adrenaline and Iloprost produced strikingly

similar time course-inhibition profiles. By contrast, the inhibition of FMLP stimulated H_2O_2 production by adenosine ($1 \times 10^{-4}M$) was most effective when cells were preincubated for 15 minutes (Fig 12).

Figure 12 - THE EFFECT OF INHIBITOR PREINCUBATION TIME ON SUBSEQUENT INHIBITION OF FMLP STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were preincubated for various time periods with adrenaline (5×10^{-5}M ●——●), Iloprost (1.4×10^{-4}M ■——■), and adenosine (1×10^{-4}M ▲——▲) in the H_2O_2 assay mixture and then stimulated with FMLP (5×10^{-6}M). Results are expressed as percent inhibition of FMLP stimulated H_2O_2 production.

(Points represent the mean values of 2 experiments)



Shorter preincubation times reduced the inhibitory activity of this agent. Inhibitor-response curves were then constructed for each stimulus/inhibitor combination employing the incubation time periods as defined by the time course studies. In addition, the selective B_2 adrenoceptor agonist salbutamol, was employed as an inhibitor for comparison with adrenaline. Like adrenaline, salbutamol most effectively inhibited H_2O_2 production when added to the cells with the stimulant. Adrenaline and salbutamol inhibited the basal and stimulated H_2O_2 responses without stimulus specificity. Comparison of ID50 values indicated that adrenaline was slightly more potent than salbutamol at inhibiting stimulated H_2O_2 production. Both agents however, were less effective at inhibiting basal H_2O_2 production (Figs 13, 14). In view of the lack of stimulus specific inhibition by adrenaline and salbutamol the inhibition profiles obtained with adenosine and Iloprost were unexpected. Adenosine inhibited basal and FMLP stimulated H_2O_2 dose dependently but no significant inhibition of the aggregated IgG response was observed until the adenosine concentration reached 10^{-4}M (Fig 15). Furthermore, adenosine was completely ineffective at inhibiting the PMA response. A similar inhibition profile was obtained with Iloprost (Fig 16). No inhibition of the aggregated IgG or PMA reponses occurred at concentrations up to 2.8×10^{-4}M. At this concentration, Iloprost abolished FMLP stimulated H_2O_2 production but was only poorly effective against basal H_2O_2 production. These results are particularly surprising in view of the fact that Iloprost can stimulate a rise in platelet cAMP and inhibit platelet aggregation in the nanomolar concentration range (Belch et al 1985).

Figure 13 - THE EFFECT OF ADRENALINE ON BASAL AND STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were incubated with the H_2O_2 assay mixture containing adrenaline at various concentrations and either FMLP (5×10^{-6}M ▲——▲) aggregated IgG (200 ug/ml ■——■) PMA (10 ng/ml ◆——◆) or buffer (for background H_2O_2 production ●——●). Results are expressed as percent H_2O_2 produced by stimulants in the absence of adrenaline.

Points represent mean of 4 experiments (standard deviation bars omitted for clarity)

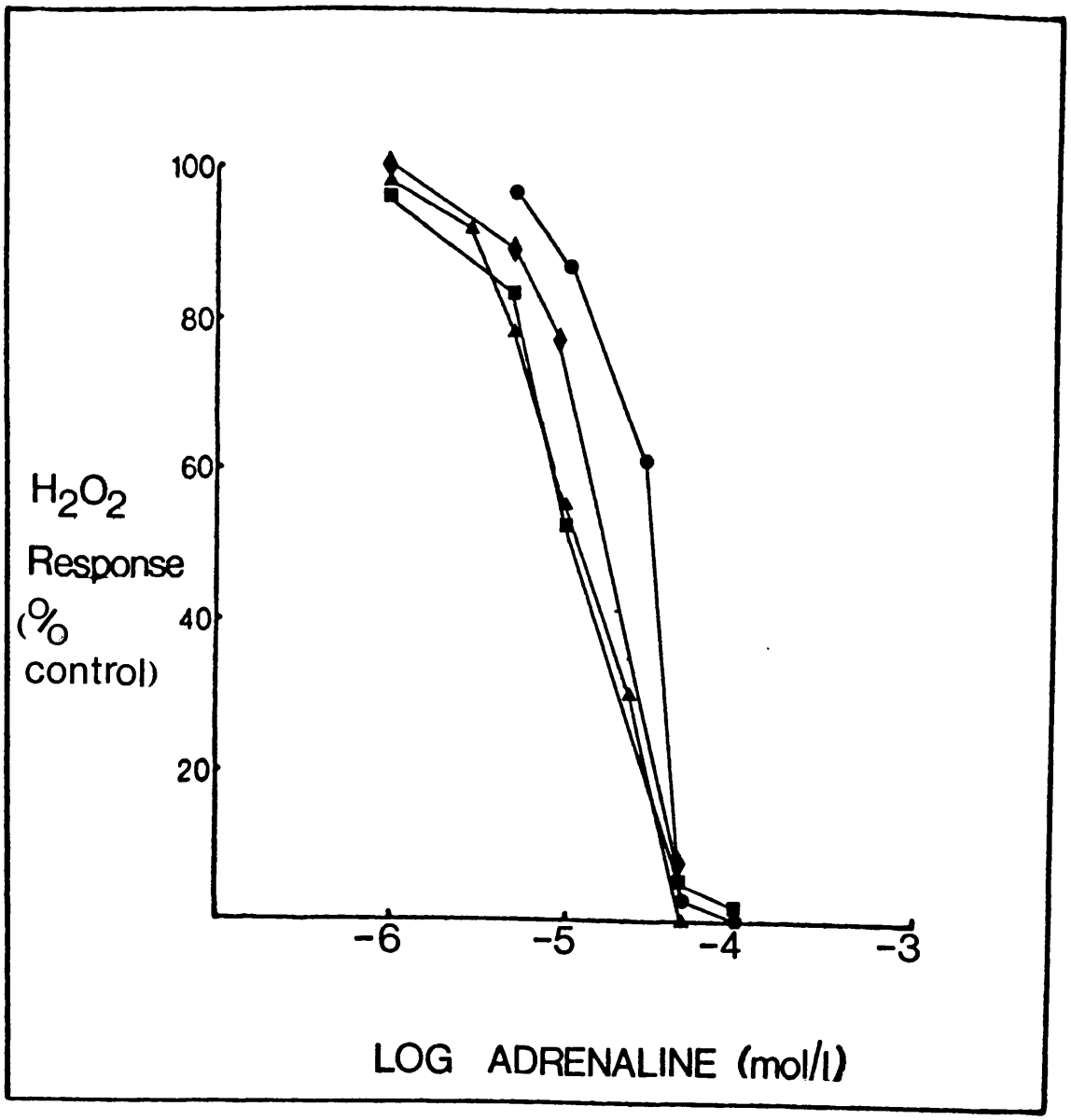


Figure 14 - THE EFFECT OF SALBUTAMOL ON BASAL AND STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were incubated with the H_2O_2 assay mixture containing salbutamol at various concentrations and either FMLP (5×10^{-6} ▲——▲) aggregated IgG (200 ug/ml■-■) PMA (10 ng/ml ◆——◆) or PBSG (for unstimulated H_2O_2 production ●——●). Results are expressed as the percent H_2O_2 production that occurred in the absence of salbutamol.

Points represent mean of 4 experiments (standard deviation bars omitted for clarity).

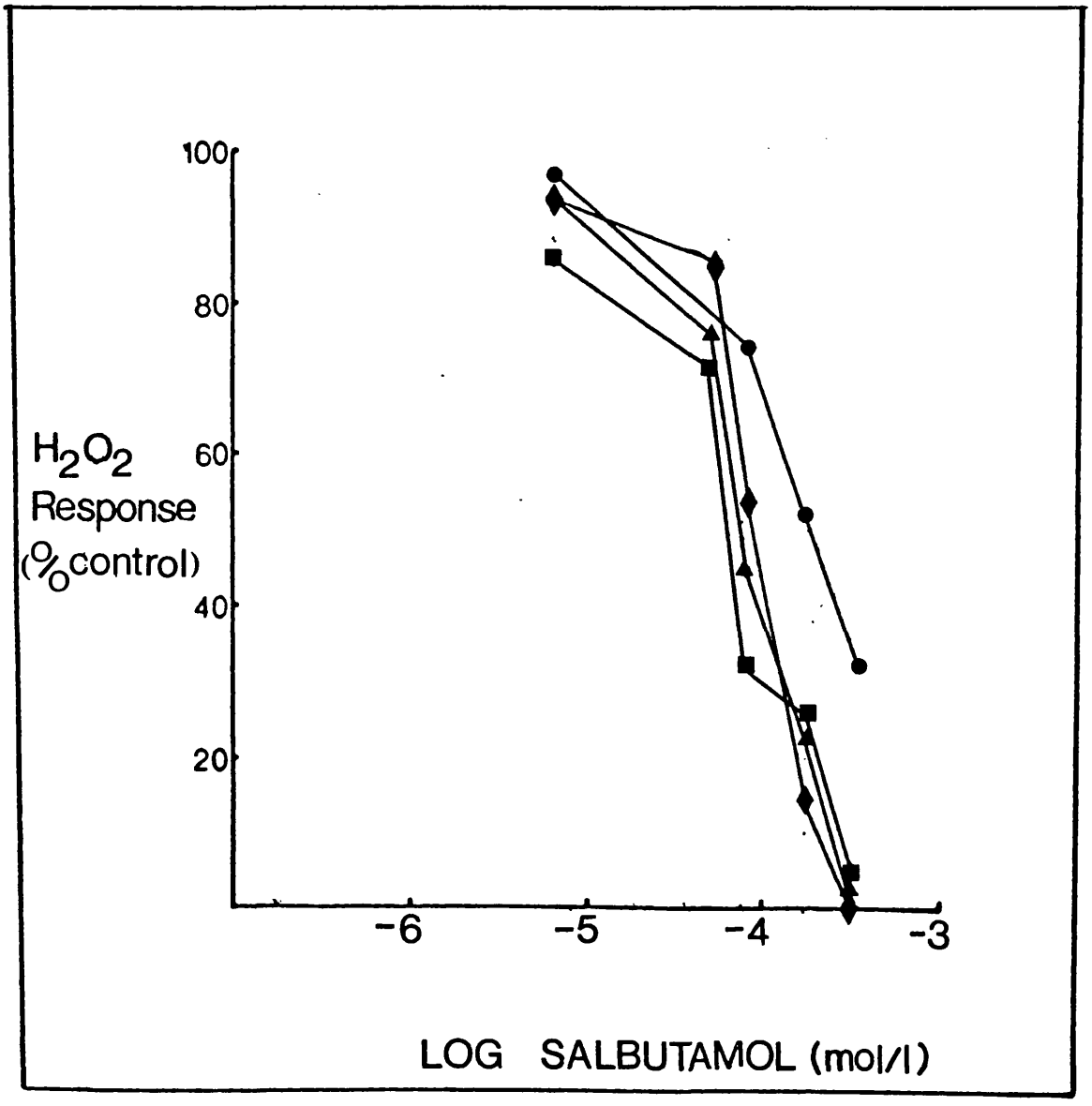


Figure 15 - THE EFFECT OF ADENOSINE ON BASAL AND STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were preincubated with adenosine for 15 minutes at 37% prior to addition to the H_2O_2 assay mixture which contained adenosine (to prevent washout phenomenon) and either FMLP ($5 \times 10^{-6}M$ ▲——▲) aggregated IgG (200 ug/ml ■——■) PMA (10 ng/ml ◆——◆) or PBSG (to measure unstimulated H_2O_2 production ●——●). Points represent mean ± standard deviation of 4 experiments

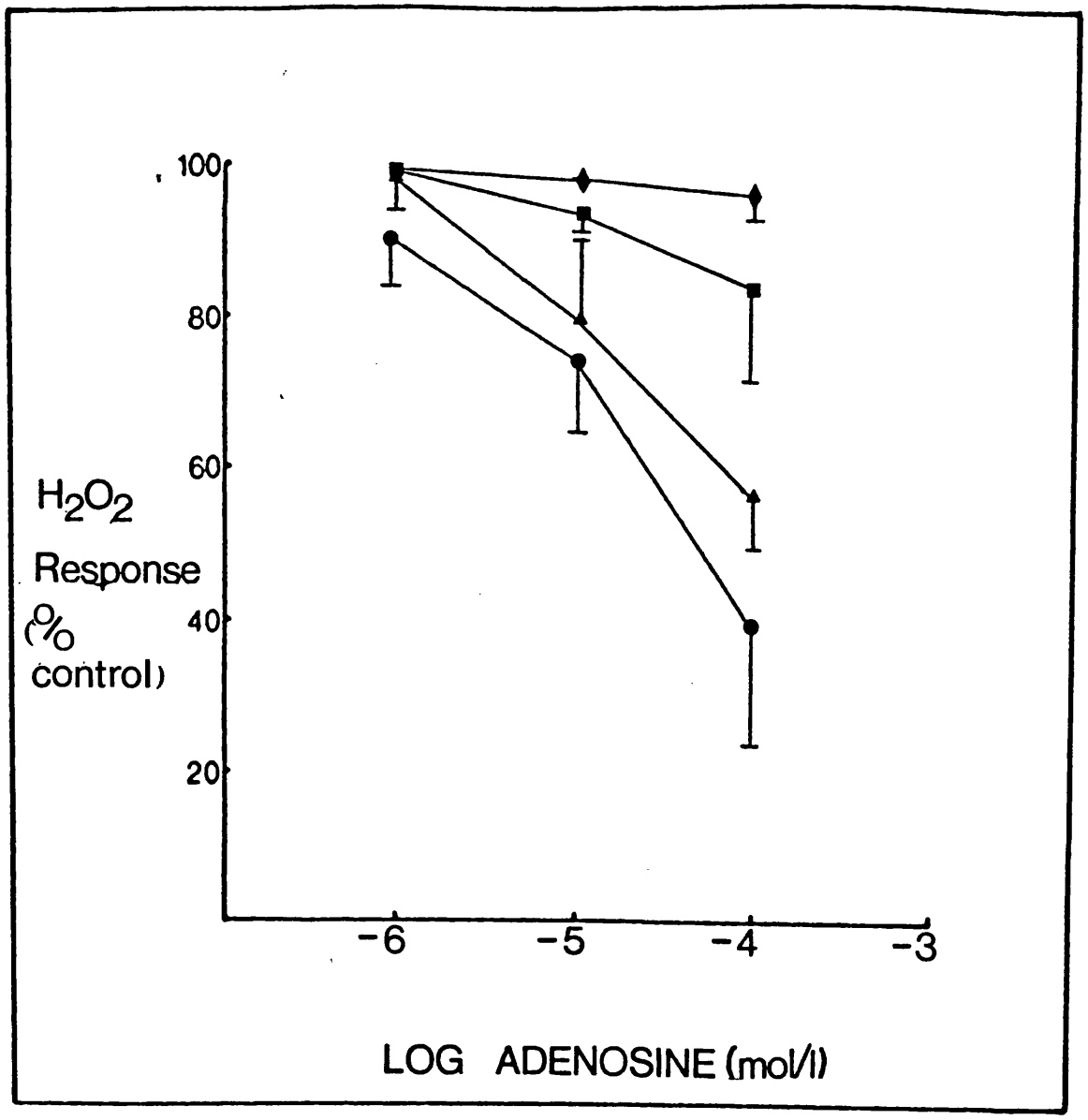
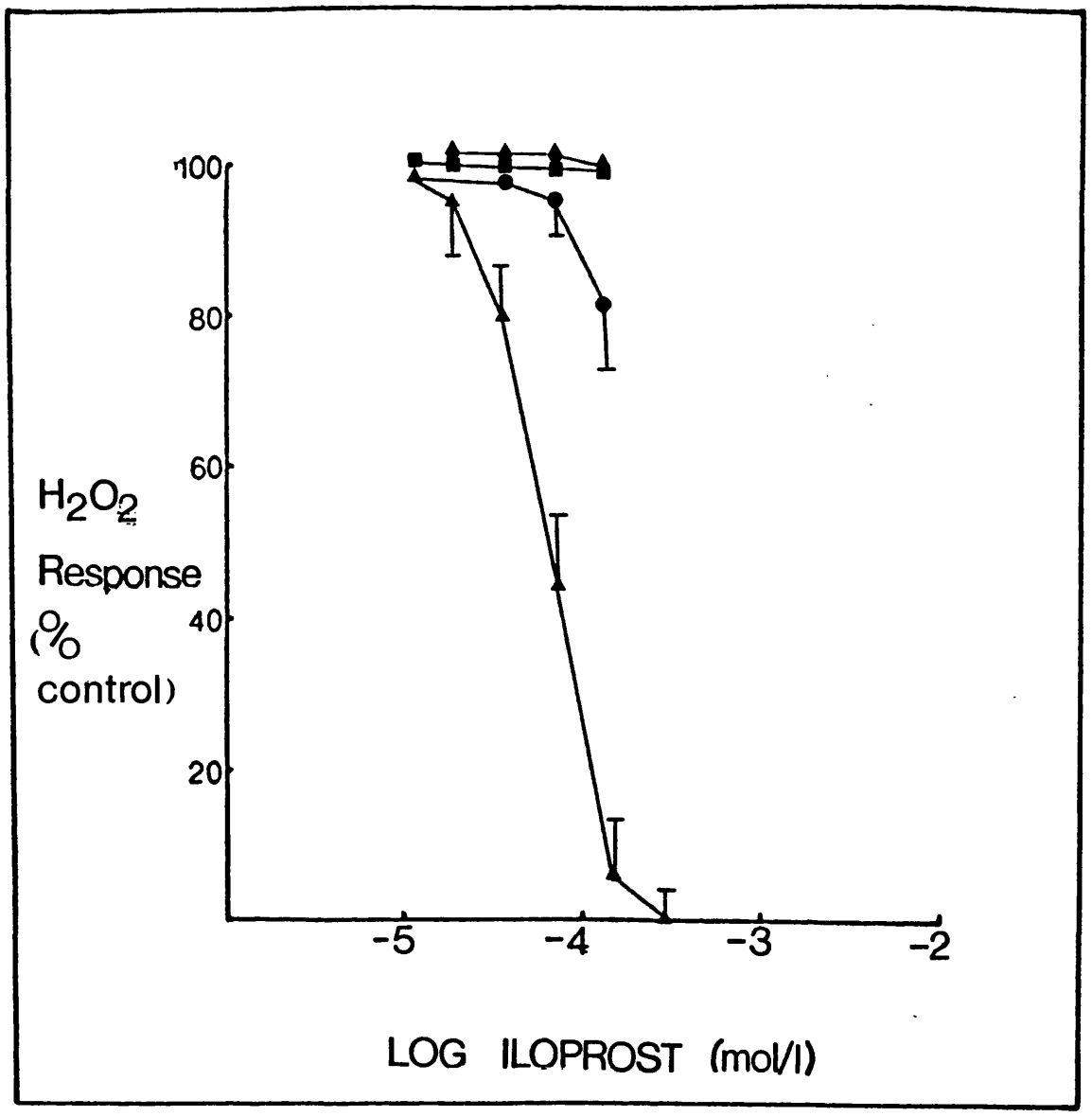


Figure 16 - THE EFFECT OF ILOPROST ON BASAL AND STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were incubated with the H_2O_2 assay mixture containing Iloprost at various concentrations and either FMLP (5×10^{-6}M ▲——▲) aggregated IgG (200 ug/ml ■-■) PMA (10 ng/ml ◆——◆) or PBSG (for unstimulated H_2O_2 production ●——●). Results are expressed as the percent H_2O_2 production that occurred in the absence of Iloprost.

Points represent mean ± standard deviation of 4 experiments



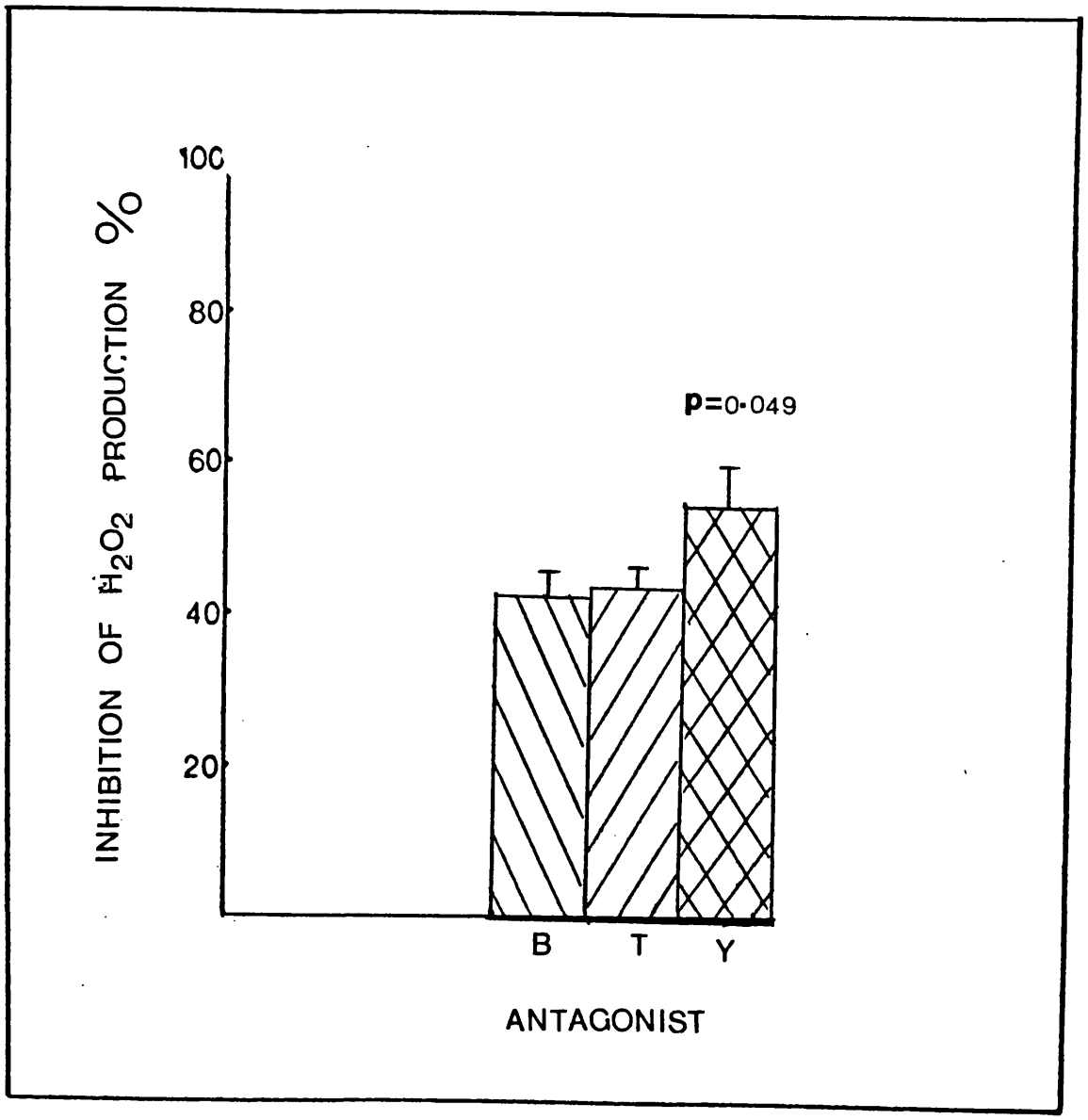
The ability of agents to stimulate α_2 adrenergic receptors and thereby inhibit agonist induced rises in intracellular cAMP is well documented (Lefkowitz 1979). α_2 receptors have been demonstrated on the surface of neutrophils by the specific binding of yohimbine to whole cell and isolated membrane preparations (Panosian and Marinetti 1983).

However the functional activity of these receptors on neutrophils has yet to be demonstrated. With this in mind the effects of yohimbine, an α_2 receptor antagonist, upon the adrenaline mediated inhibition of H_2O_2 production was investigated. Adrenaline stimulates both B_2 and α_2 receptors. Since the stimulation of B_2 receptors results in an elevation of intracellular cyclic AMP and α_2 receptor stimulation inhibits this response it would be expected that the blockade of α_2 receptors would enhance the adrenaline stimulated rise in cAMP and consequently its inhibitory effect on neutrophil H_2O_2 production. Fig 17 shows the effects of preincubating neutrophils with 5×10^{-5}M yohimbine for 15 minutes prior to stimulation with FMLP in the presence of adrenaline. A small but significant increase in adrenaline mediated inhibition was observed ($p < 0.05$). Preincubation with yohimbine followed by stimulation with FMLP alone had no effect upon the subsequent H_2O_2 response. These observations indicate that not only are $_2$ receptors present on the neutrophil cell membrane but they are in fact functional. Preincubation of neutrophils for 15 minutes with timolol (5×10^{-5}M) a selective B_1 adrenoceptor antagonist had no effect upon the subsequent adrenaline mediated inhibition of FMLP stimulated H_2O_2 production. This is not surprising in view of a report that B receptors on neutrophils are confined to the B_2 subtype (Gallant and Alfred 1980).

Where agents inhibit the oxidative responses of neutrophils regardless of the stimulus employed it is always advisable to investigate the possibility of a direct anti-oxidant effect. This has been carried out in these studies by the following method. Superoxide anion was generated artificially using a xanthine/xanthine oxidase system (Dixit et al 1982). The superoxide

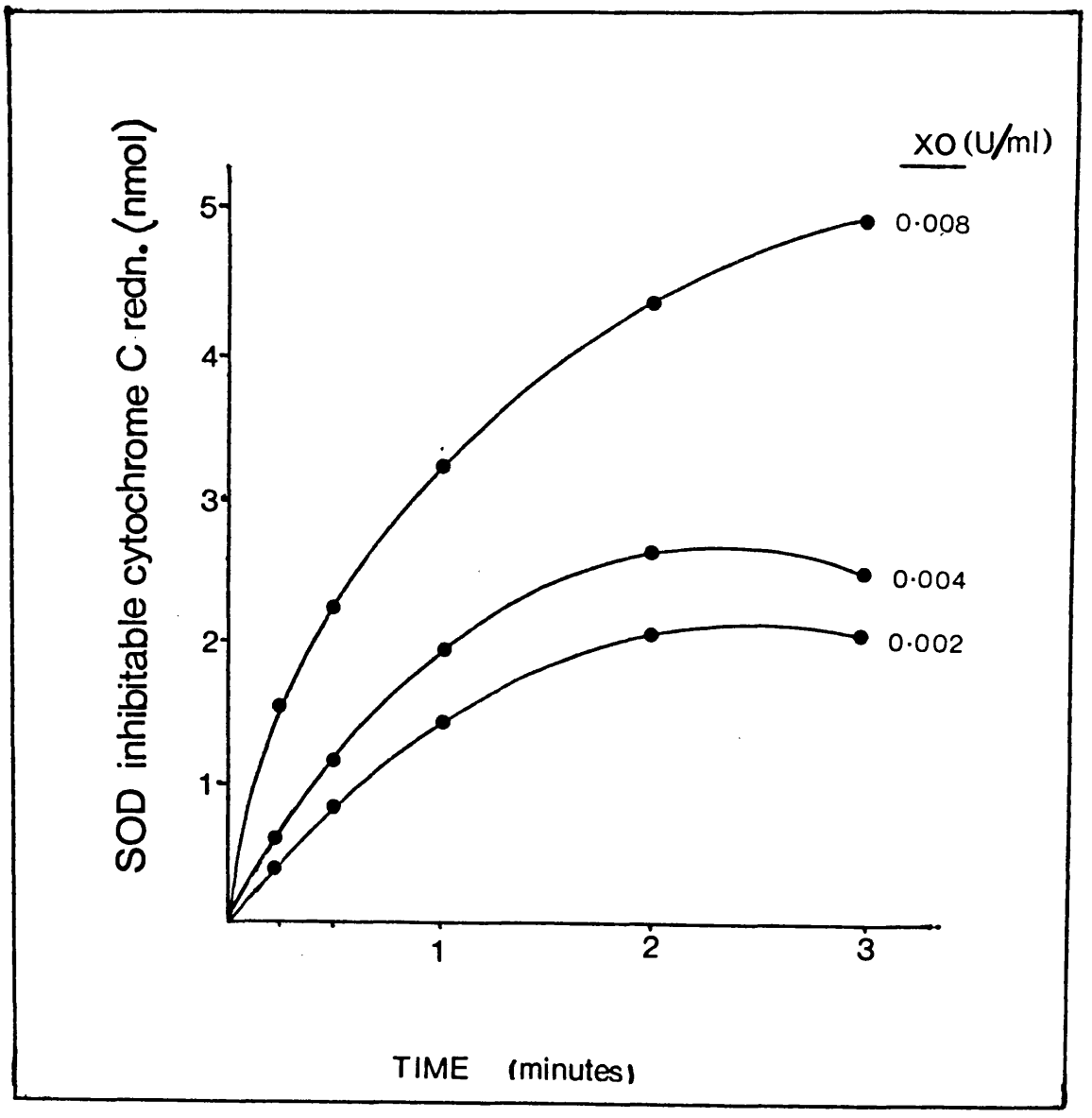
Figure 17 - THE EFFECT OF ADRENOCEPTOR BLOCKADE ON THE ADRENALINE MEDIATED INHIBITION OF FMLP STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were preincubated for 15 minutes at 37°C with yohimbine, (Y 5 x 10^{-5}M) timolol, (T 5 x 10^{-5}M) or PBSG (buffer) and then added to the H_2O_2 assay mixture containing adrenaline (5 x 10^{-5}M) and inhibitor (to prevent washout) and FMLP (5 x 10^{-6}M). Results are expressed as percent inhibition of FMLP stimulated H_2O_2 production (mean + SD of 3 experiments).



generated was detected by its ability to reduce cytochrome C, and confirmed by inhibiting the reaction with superoxide dismutase. Agents that possess direct antioxidant activity inhibit the reduction of cytochrome C. Fig 18 shows the effect of various concentrations of xanthine oxidase in the presence of a saturated xanthine solution, on cytochrome C reduction.

Figure 18 - THE REDUCTION OF CYTOCHROME C BY SUPEROXIDE GENERATED BY XANTHINE/XANTHINE OXIDASE

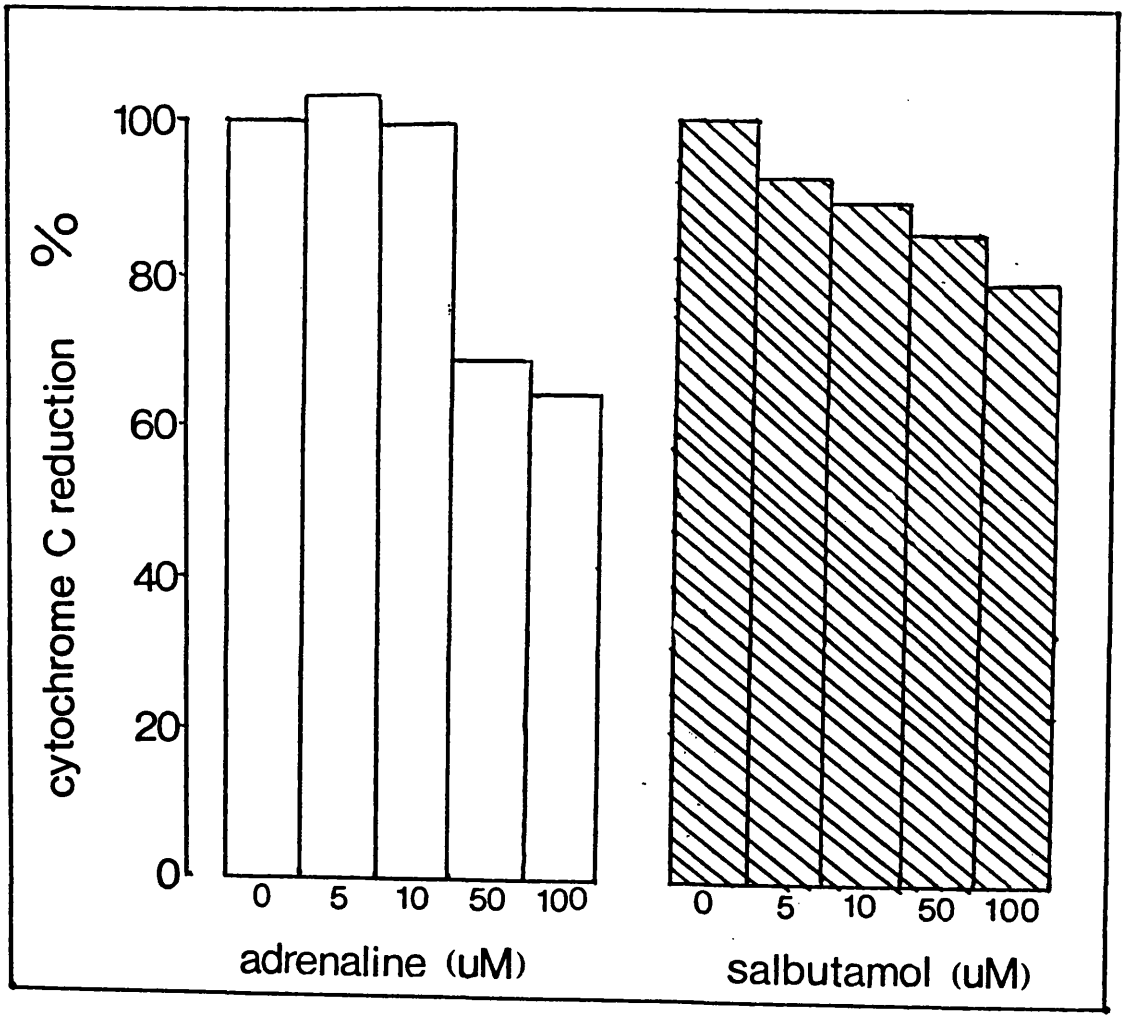


Both the rate and magnitude of cytochrome C reduction increased with increasing concentrations of xanthine oxidase. To investigate the direct antioxidant activity of adrenaline and salbutamol, these agents were incubated with the superoxide generating system (0.008 U/ml xanthine oxidase) to see whether the reduction of cytochrome C was inhibited. In a two experiments adrenaline (5×10^{-5}M) and salbutamol (1×10^{-4}M) inhibited cytochrome C reduction by 30% and 20% respectively (Fig 19). Although significant, the direct antioxidant activity of these agents contributed in only a minor way to the inhibitory effects of these agents upon neutrophil H_2O_2 production (100% at the indicated concentrations).

Figure 19 - THE EFFECTS OF ADRENALINE AND SALBUTAMOL ON CYTOCHROME C REDUCTION BY SUPEROXIDE GENERATED BY XANTHINE OXIDASE/XANTHINE.

Results are expressed as the % cytochrome C reduction that occurred in the absence of the two inhibitors.

Bars represent the mean of 2 experiments.



The Effects of Intravenous Iloprost Infusion Upon Basal and Stimulated H_2O_2 Production

In a double blind cross over study patients with Raynauds Phenomenon associated with systemic sclerosis were administered either Iloprost (up to 20 ng/kg/min) or placebo for six hours per day over three successive days. Neutrophils were obtained and purified as described in Materials and Methods before and after treatment and basal and FMLP stimulated H_2O_2 production were measured.

After a six week clinical assessment and washout period the patients crossed over to the alternative treatment and the procedure described above was repeated. The assessment of neutrophil activity was carried out blind (Fig 20-23). Neither placebo nor Iloprost infusion significantly affected basal or FMLP stimulated H_2O_2 production. Neutrophils from some patients, however, did appear to be less inactive following Iloprost therapy but this was not associated with any signs of clinical movement.

Figure 20 - UNSTIMULATED HYDROGEN PEROXIDE PRODUCTION IN SS NEUTROPHILS BEFORE AND AFTER INTRAVENOUS PLACEBO INFUSION

Each point represents the mean of 2 determinations for each patient. H_2O_2 production is expressed in umol/l/10^6 cells/30 minutes.

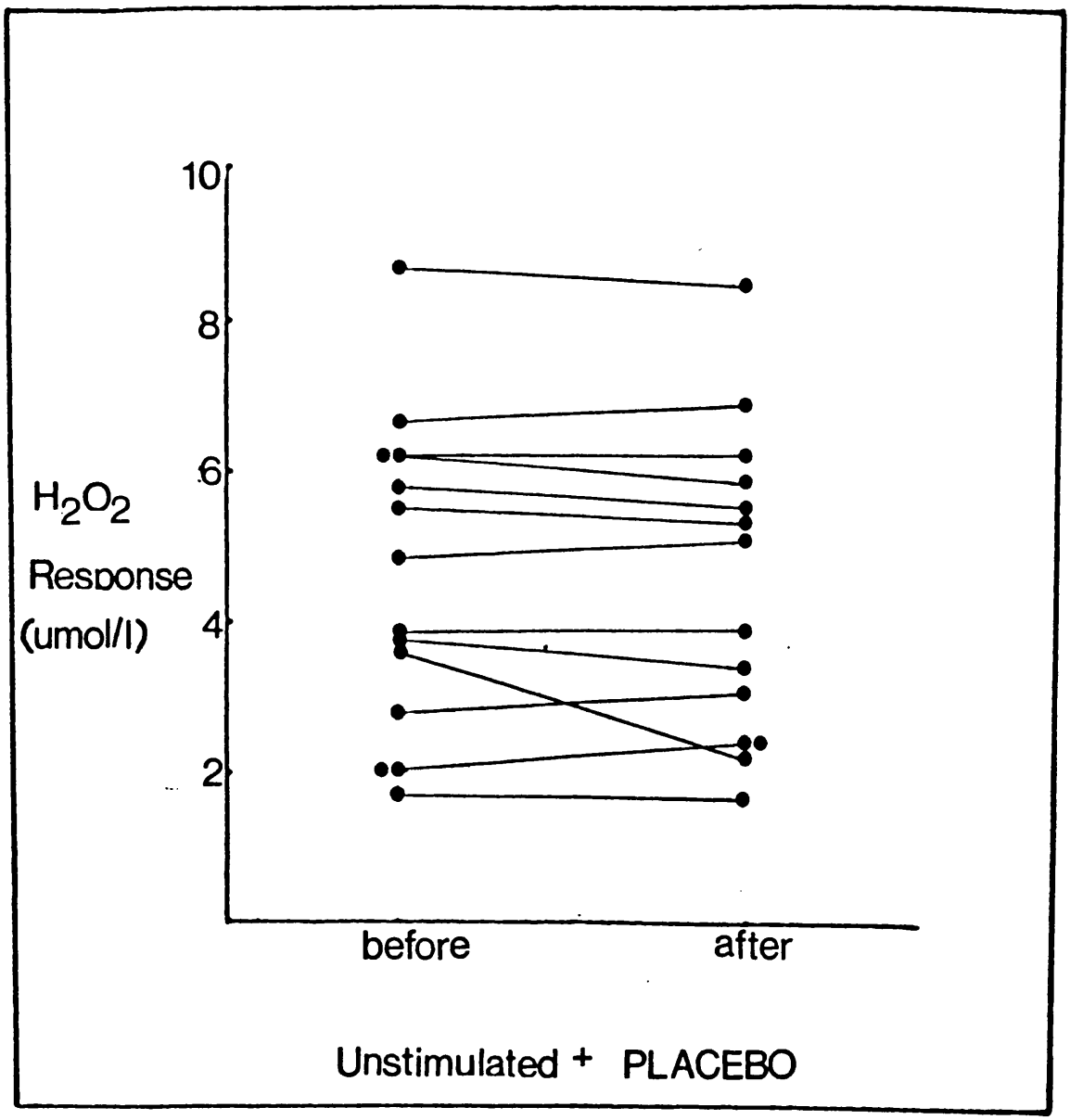


Figure 21 - UNSTIMULATED HYDROGEN PEROXIDE PRODUCTION IN SS NEUTROPHILS BEFORE AND AFTER INTRAVENOUS ILOPROST INFUSION

Each point represents the mean of 2 determinations for each patient. H_2O_2 production is expressed in umol/l/10^6cells/30 minutes.

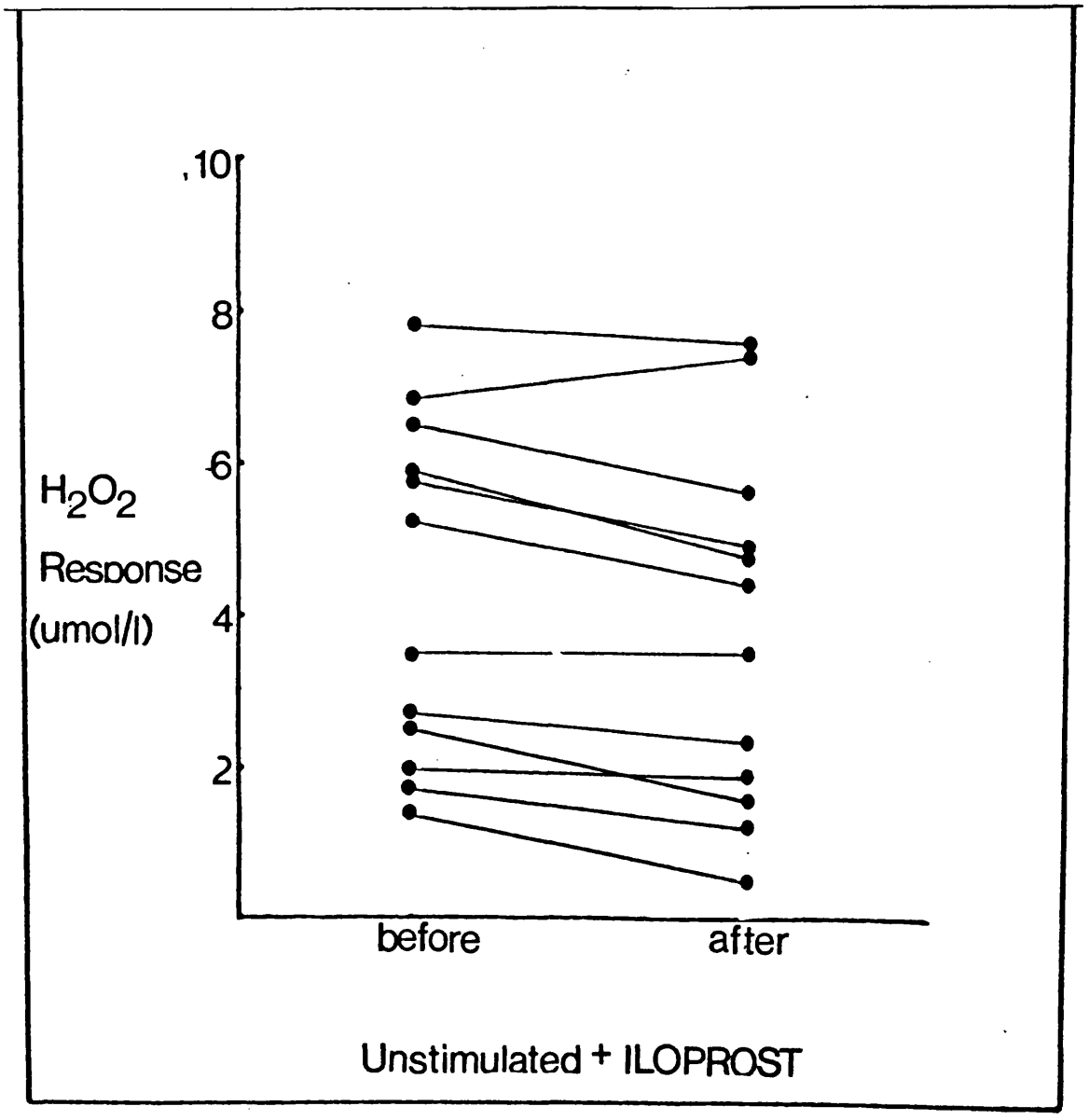


Figure 22 - FMLP STIMULATED HYDROGEN PEROXIDE PRODUCTION IN SS NEUTROPHILS BEFORE AND AFTER INTRAVENOUS PLACEBO INFUSION

Each point represents the mean of 2 determinations for each patient. H_2O_2 production is expressed in umol/l/10^6cells/30 minutes.

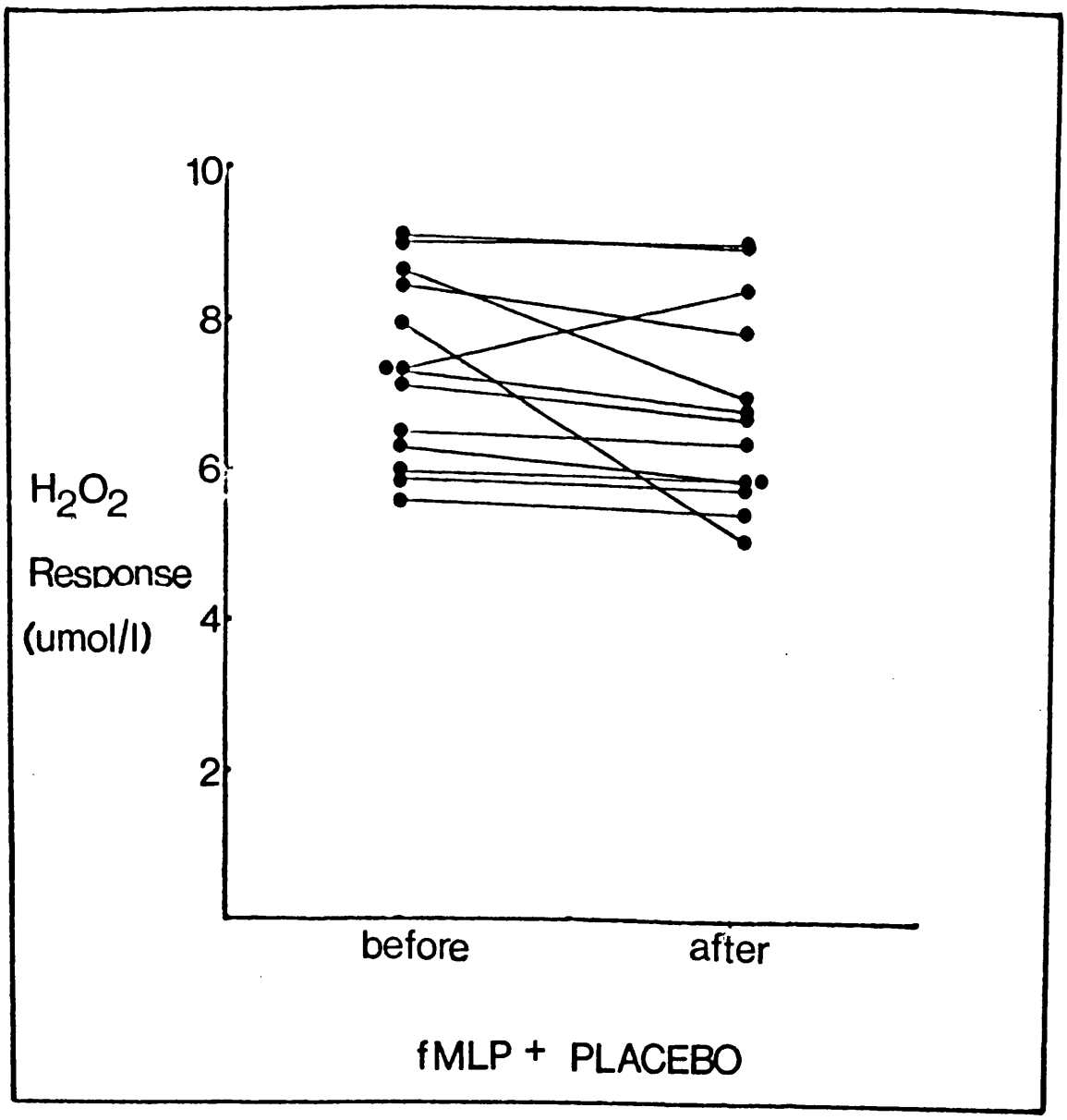
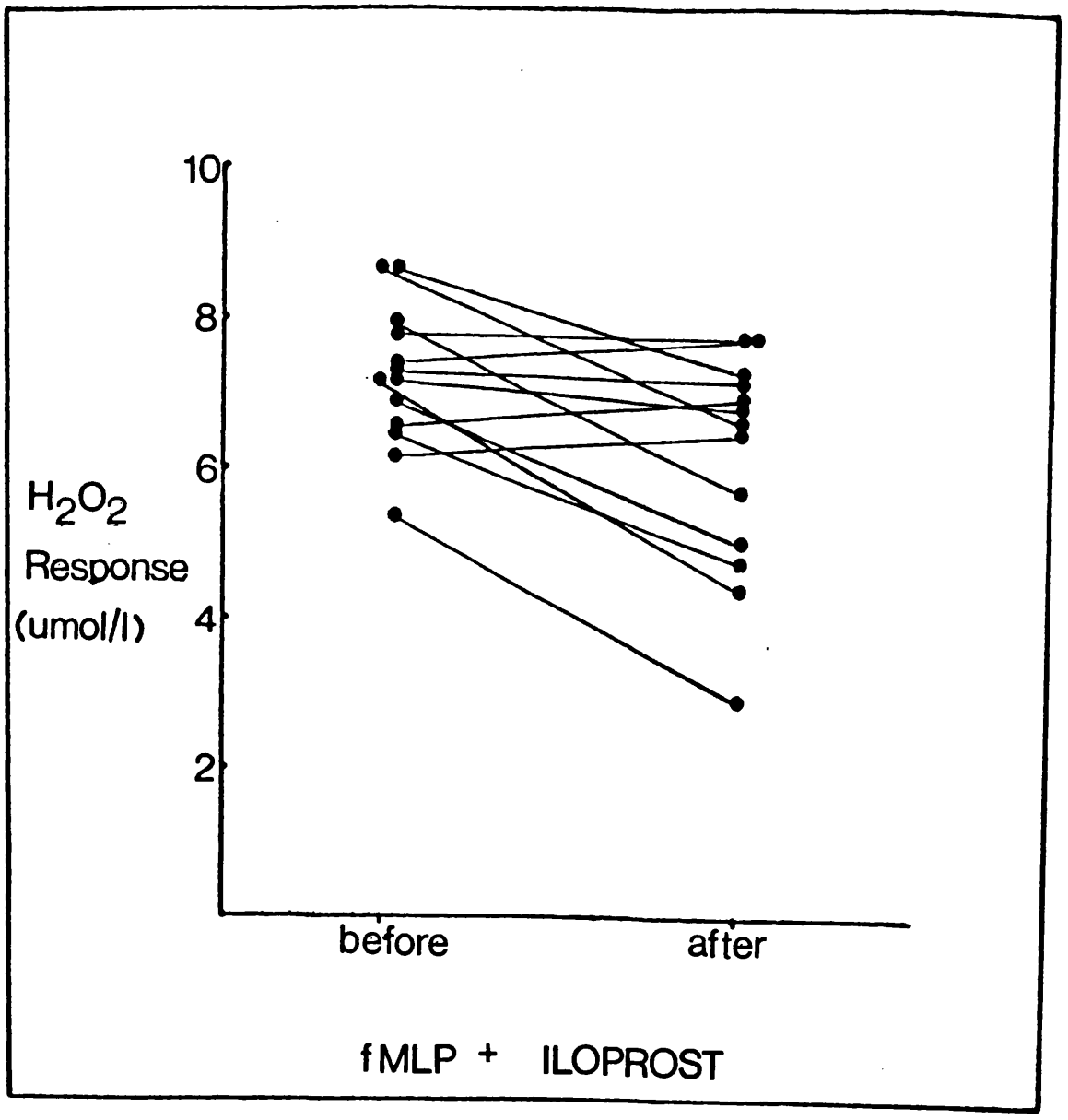


Figure 23 - FMLP STIMULATED HYDROGEN PEROXIDE PRODUCITON IN SS NEUTROPHILS BEFORE AND AFTER INTRAVENOUS ILOPROST INFUSION

Each point represents the mean of 2 determinations for each patient. H_2O_2 production is expressed in umol/l/10^6cells/30 minutes.



CHAPTER 5

The Role of Phospholipase A_2 in Neutrophil Signal Transduction: Studies Employing Human Recombinant Lipocortin

Within the neutrophil plasma membrane are sites for the reception, modulation and translation of extracellular signals into functional cellular responses. Recently membrane phospholipids have been implicated in transmembrane signalling. For example the phospholipase C catalysed breakdown of PIP_2 that accompanies neutrophil stimulation by FMLP correlates closely with both receptor binding and the magnitude of the final response (Takenawa et al 1985). Phospholipase A_2 may also be important in transmembrane signalling (Maridonneau-Parini et al 1986). Hydrocortisone, a corticosteroid anti-inflammatory drug which inhibits PLA_2 through the directed synthesis of lipocortin, also inhibits neutrophil chemotaxis and the release of arachidonic acid (Hirata et al 1980). In the following studies the effects of human recombinant lipocortin on the chemotactic, polarization and oxidative responses of neutrophils were investigated and compared with two synthetic PLA_2 inhibitors; mepacrine and parabromophenacylbromide (pBPB).

The Effect of PLA_2 Inhibition on Neutrophil Oxidative Metabolism

The effects of r-lipocortin, mepacrine and pBPB upon resting and stimulated H_2O_2 production were investigated. Time course studies revealed that a fifteen minute cell preincubation period with all agents was required for the optimal inhibition of FMLP stimulated H_2O_2 production (Fig 24).

Both the inhibition by lipocortin and mepacrine were reversible as assessed by washout phenomena. In contrast, the inhibition of FMLP stimulated H_2O_2 production by pBPB was not affected by washout (Table 3). This observation is in accordance with those of Roberts et al (1976) who suggest that irreversible acetylation of a histidine residue on PLA_2 by pBPB is responsible for the loss of enzyme activity.

Table 3

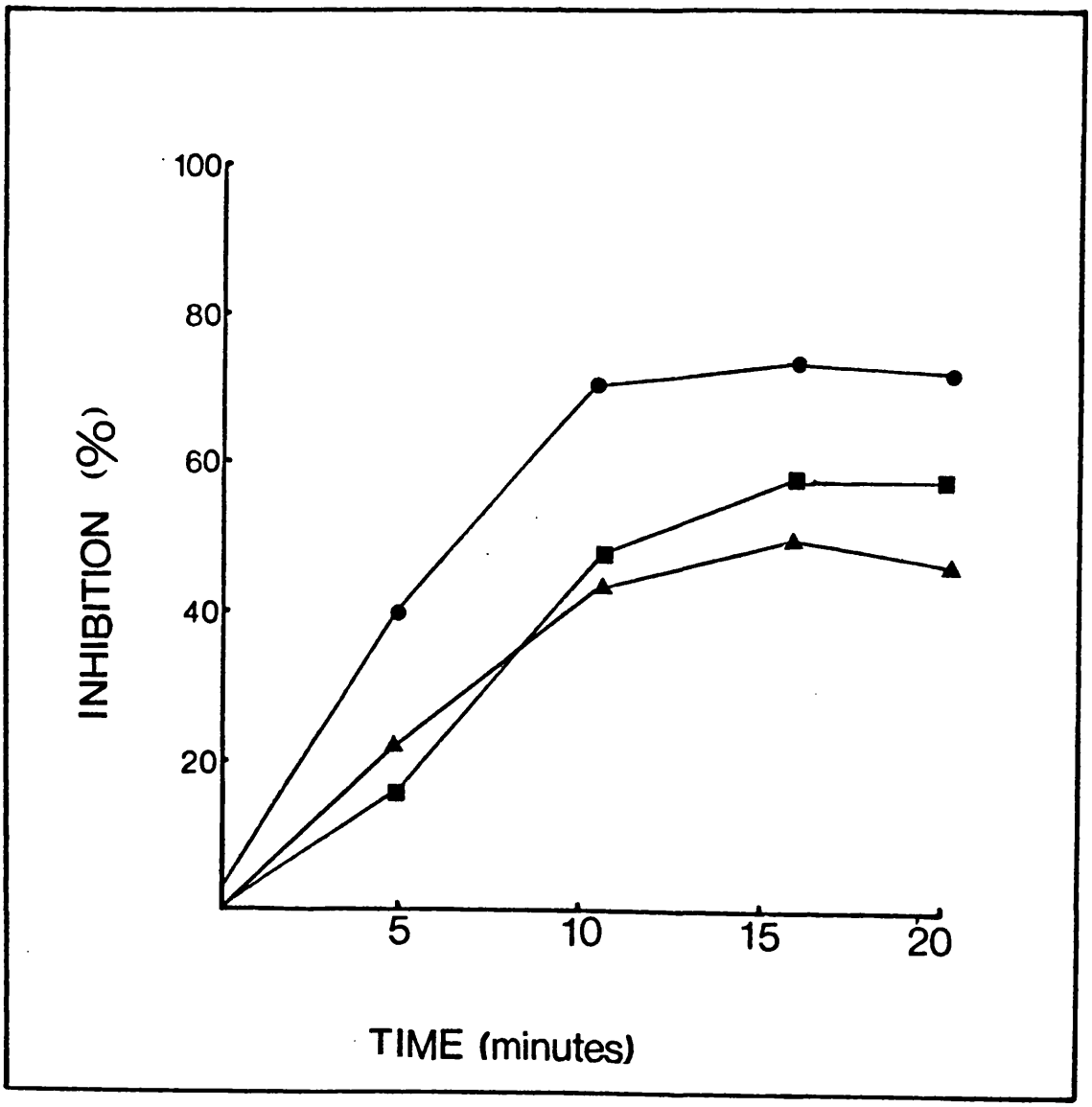
The effect of washout on the inhibition of FMLP stimulated hydrogen peroxide production by PLA_2 inhibitors.

	% INHIBITION	
INHIBITOR	WITH INHIBITOR	WITH BUFFER
RECOMBINANT LIPOCORTIN (5×10^{-6}M)	34.4	0.9
MEPACRINE (1×10^{-4}M)	87.3	0.0
pBPB (5×10^{-6}M)	61.5	67.2

Figure 24 - THE EFFECT OF INHIBITOR PREINCUBATION TIME ON SUBSEQUENT INHIBITION OF FMLP STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were preincubated with lipocortin (1×10^{-5} M - ●——●) pBPB (5×10^{-6}M ■——■) and mepacrine (1×10^{-4}M - ▲——▲) and then added to the H_2O_2 assay mixture containing inhibitor (to prevent washout) and FMLP (5×10^{-6}M). Results are expressed as % inhibition of FMLP stimulated hydrogen peroxide production.

Points represent the mean of 2 experiments.



Utilising a standardised 15 minutes preincubation period the effect of PLA_2 inhibitors on the oxidative responses to heat aggregated IgG (200 ug/ml) FMLP (5 x 10^{-6}M) and phorbol myristate acetate (10 ng/ml) were investigated. Both pBPB and recombinant lipocortin showed similar inhibition profiles with regard to the stimulus employed. Lipocortin effectively inhibited both basal H_2O_2 production and that stimulated by FMLP (Fig 25). The responses to PMA and aggregated IgG were not significantly inhibited at even the highest concentrations of lipocortin (1 x 10^{-5}M) ($p > 0.05$). Likewise pBPB effectively inhibited both basal and FMLP stimulated H_2O_2 production (Fig 26). At higher concentrations (10^{-5}M) the aggregated IgG response was also partially inhibited. Comparison of ID50 values revealed that pBPB was about 5 x more potent at inhibiting FMLP and basal responses than the aggregated IgG response (Table 4). The PMA response was relatively insensitive to pBPB inhibition. At concentrations greater than 1 x10^{-5}M the cytotoxicity of pBPB emerged (>20% at 5 x 10^{-5}M). No cytotoxicity occurred at any of the employed concentrations of lipocortin or mepacrine. The degree of inhibition by pBPB of the FMLP stimulated oxidative response was similar to that reported by other workers (Smolen and Weissman 1980, Neal et al 1987) and was in the concentration range reported to inhibit PLA_2 activity (Duque et al 1986).

The effects of mepacrine contrasted with those of pBPB and lipocortin. Although basal H_2O_2 production was effectively inhibited in a dose dependent manner by mepacrine, its inhibitory effects upon FMLP stimulated H_2O_2 were extremely poor in comparison. Furthermore both

HAGG and PMA responses were inhibited (Fig 27). A summary of the data from Fig 25-27 is shown in Table 4.

Figure 25 - THE EFFECT OF HUMAN RECOMBINANT LIPOCORTIN ON BASAL AND STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were preincubated for 15 minutes at 37°C with human recombinant lipocortin and then added to the H_2O_2 assay mixture containing lipocortin (to prevent washout phenomenon) and either FMLP (5×10^{-6}M ▲——▲) aggregated IgG (200 ug/ml -■——■) PMA (10 ng/ml ◆——◆) or PBSG (for unstimulated H_2O_2 production ●——●). Results are expressed as the percent H_2O_2 produced by each stimulant in the absence of lipocortin.

Each point represents the mean ± standard deviation of at least 4 experiments.

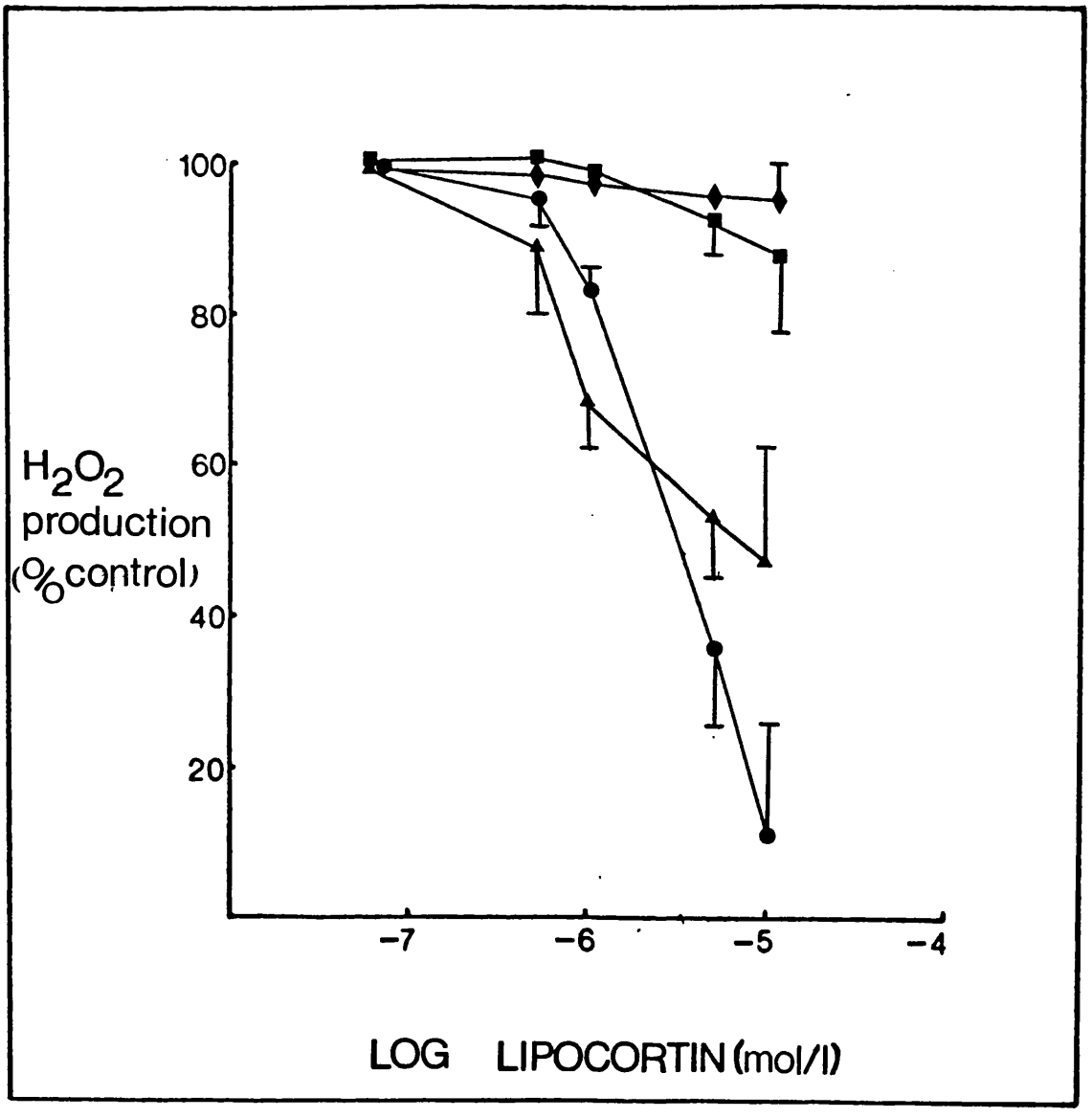


Figure 26 - THE EFFECTS OF PARABROMOPHENACYLBROMIDE ON BASAL AND STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were preincubated for 15 minutes at 37°C with pBPB and then added to the H_2O_2 assay mixture containing either FMLP (5 x 10^{-6}M ▲——▲) aggregated IgG (200 ug/ml ■——■) PMA (10 ng/ml ◆——◆) or PBSG (to measure unstimulated H_2O_2 production ●——●). Results are expressed as the percent H_2O_2 produced by each stimulant in the absence of pBPB.

Each point represents the mean ± standard deviation of at least 3 experiments.

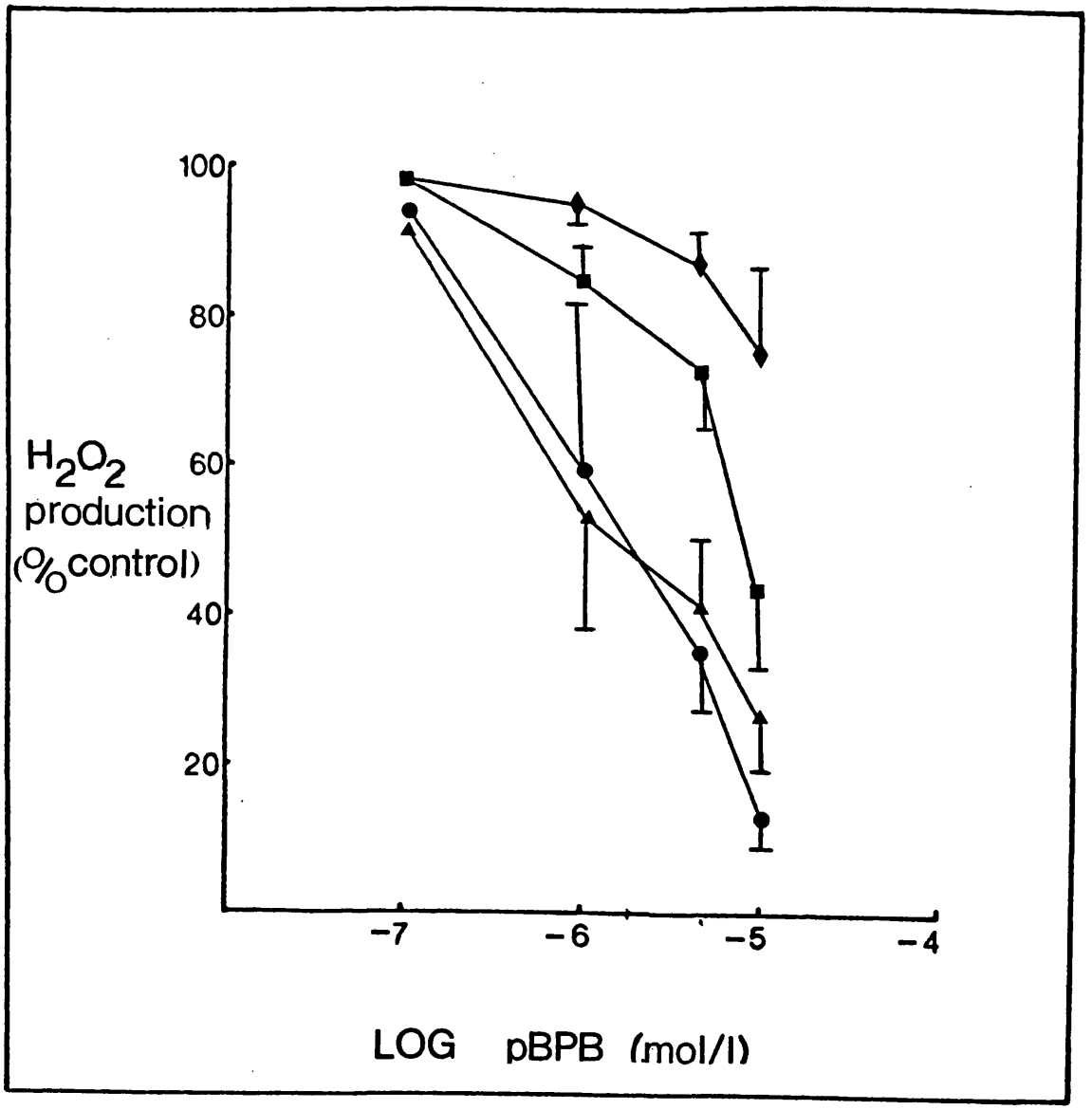


Figure 27 - THE EFFECT OF MEPACRINE ON BASAL AND STIMULATED HYDROGEN PEROXIDE PRODUCTION

Human neutrophils were pre-incubated for 15 minutes at 37°C with mepacrine and then added to the H_2O_2 assay mixture containing mepacrine (to prevent wash-out phenomenon) and either FMLP (5×10^{-6}M ▲——▲) aggregated IgG (200 ug/ml ■——■) PMA (10 ng/ml ◆——◆) or PBSG (to measure unstimulated H_2O_2 production ●——●). Results are expressed as the percent H_2O_2 produced by each stimulant in the absence of mepacrine.

Each point represents the mean ± standard deviation of at least 3 experiments.

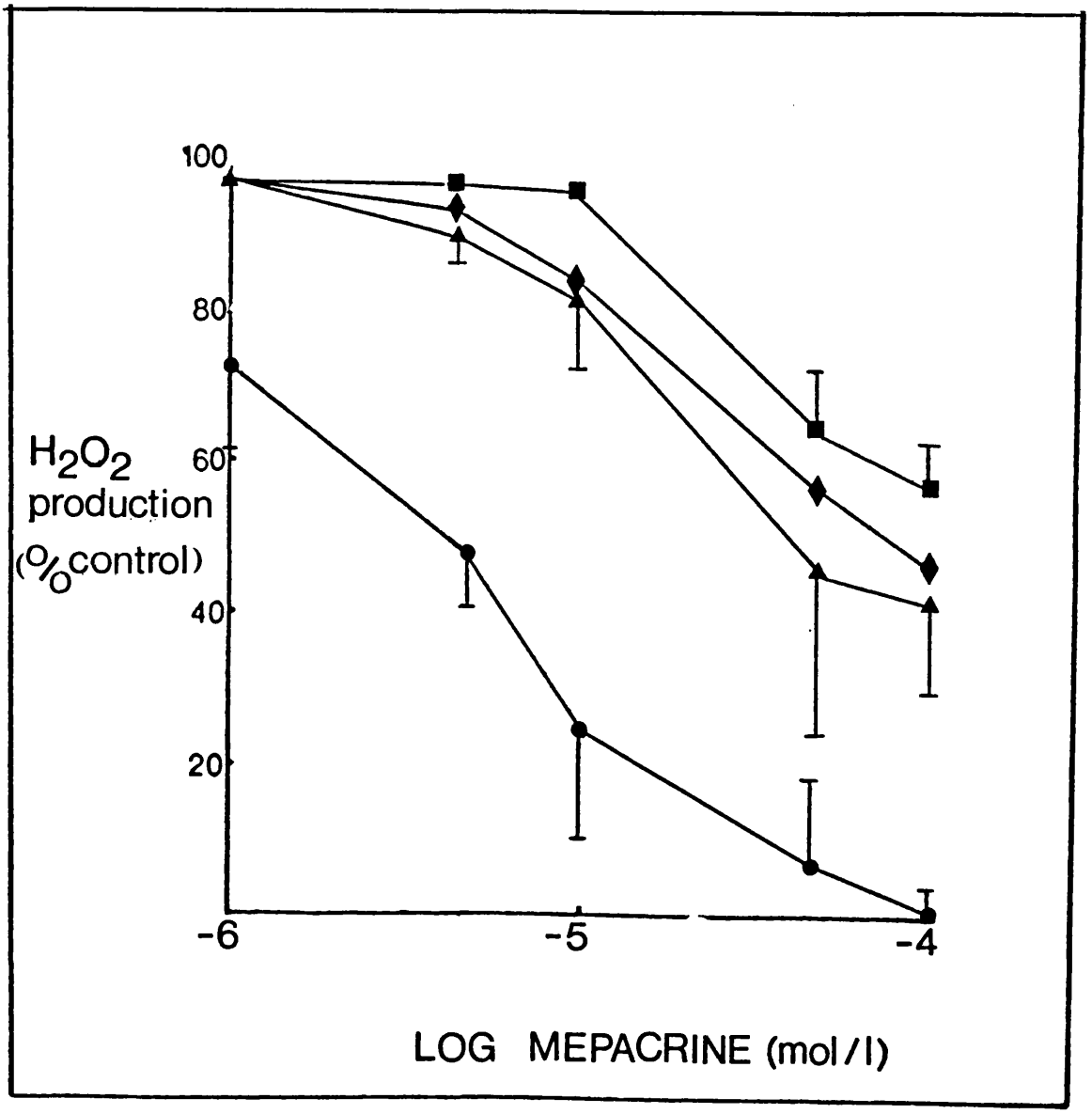


Table 4

The effect of phopholipase A_2 inhibitors on basal and stimulated hydrogen peroxide production. Values expressed are for (A) the concentration of inhibitor required to reduce the hydrogen peroxide response by 50% (ID_{50}) and (B) the maximum percent inhibition (MI%).

A ID_{50}	REST	fMLP	IgG	PMA
LIPOCORTIN	$3{\cdot}4\times10^{-6}$	$1{\cdot}0\times10^{-5}$		
pBPB	$1{\cdot}8\times10^{-6}$	$1{\cdot}8\times10^{-6}$	$9{\cdot}6\times10^{-6}$	
MEPACRINE	$5{\cdot}0\times10^{-6}$	$5{\cdot}0\times10^{-5}$		$1{\cdot}0\times10^{-4}$

B MI%	REST	fMLP	IgG	PMA
LIPOCORTIN	83·4	53·6	14·6	4·9
pBPB	88·0	73·2	55·0	22·8
MEPACRINE	100·0	57·0	40·0	51·0

The Effect of PLA_2 Inhibitors on the Basal and Stimulated Release of ^{14}C Arachidonic Acid in Neutrophils

Neutrophils were labelled with ^{14}C arachidonic acid (0.5 uCi/5 x 10^7 cells) and then washed once in PBSG/HSA and once in PBSG. The cells were then preincubated with/without inhibitors for 15 minutes at 37°C using concentrations effective in the H_2O_2 assay. Following this, either buffer (to measure background release) or the stimulants were added and incubated at 37°C for 15 minutes. The reaction was then stopped by rapid centrifugation in an Eppendorf centrifuge. Aliquots of 200 ul of the supernatant were added to 3.8 mls of Optiphase safe scintillation fluid and counted for reactivity in a liquid scintillation counter. Arachidonic acid uptake as a function of time was measured by preincubating the cells for various time intervals with ^{14}C-AA and then stopping the incorporation by rapid centifugation. The activity of the supernatant was then compared with that of a blank tube containing the same volume of buffer and ^{14}C-AA but no cells.

^{14}C arachidonic acid uptake was maximal after 15 minutes preincubation time. Fig 28 demonstrates a typical uptake graph. However, the maximum uptake of the radiolabel by neutrophils from different subjects varied considerably (between 30 and 80%).

Basal ^{14}C arachidonic acid release was consistent (7.3% 0.55) between different neutrophil preparations and similar to that reported by other workers (Dr L Steel, BootsCo plc, personal communication). However, in contrast to previous reports FMLP stimulated only minimal ^{14}C-AA release (2.8% at 5 x 10^{-6}M FMLP) (Fig 29) and aggregated IgG did not

increase release over basal levels. As expected, PMA did not stimulate the release of arachidonic acid (Maridonneau-Parini et al 1986).

Figure 28 - UPTAKE OF ^{14}C ARACHIDONIC ACID BY HUMAN NEUTROPHILS AS A FUNCTION OF TIME

Results are expressed as the ^{14}C-AA taken up by neutrophils as a percent of the amount added to the cells.

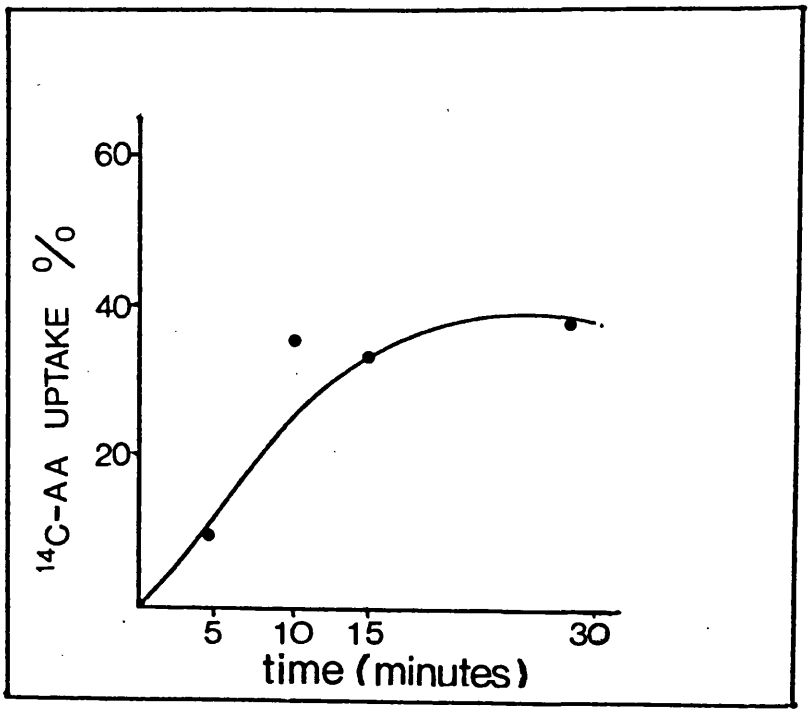
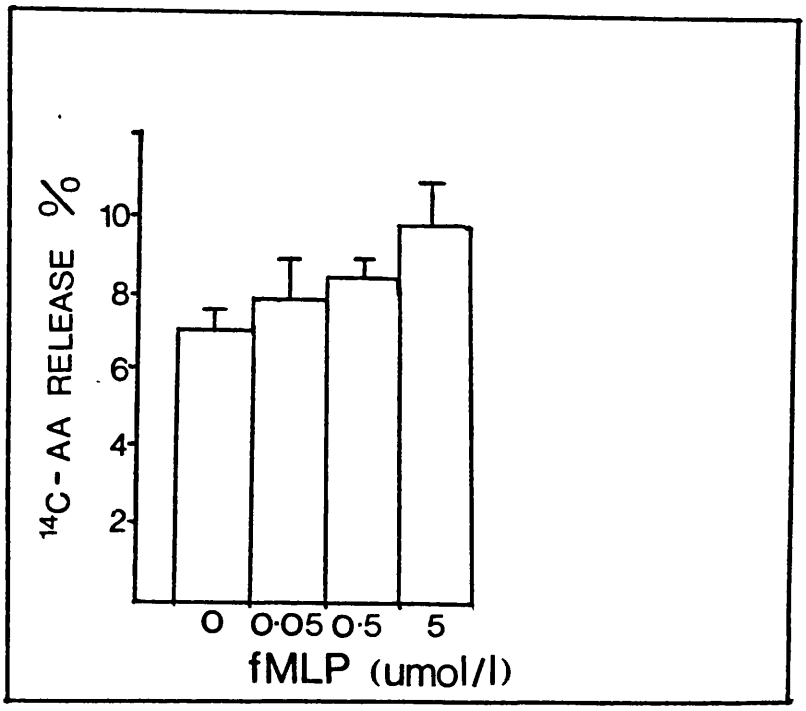


Figure 29 - BASAL AND FMLP STIMULATED ^{14}C ARACHIDONIC ACID RELEASE IN HUMAN NEUTROPHILS

Results are expressed as ^{14}C-AA release as a percent of that taken up by the cells.

Each bar represents the mean ± standard deivation of 3 experiments.



In view of the low levels of ^{14}C arachidonic acid release stimulated by FMLP ($5 \times 10^{-6}M$) (which amounted to an average release of 400 CPM over basal) the effects of the three phospholipase A_2 inhibitors on spontaneous ^{14}C arachidonic release were investigated. At concentrations employed to inhibit neutrophil hydrogen superoxide production neither lipocortin, pBPB or mepacrine inhibited spontaneous ^{14}C arachidonic acid release (Table 5).

Table 5

The effects of phopholipase A_2 inhibitors on basal ^{14}C arachidonic acid release.

PLA_2 INHIBITOR	CONCENTRATION	14C-AA RELEASE % Mean ± SD (n)
BUFFER		7.3 ± 0.55 (3)
LIPOCORTIN	(1×10^{-5}M)	7.5 ± 0.27 (3)
pBPB	(5×10^{-6}M)	7.3 ± 0.86 (3)
MEPACRINE	(1×10^{-4}M)	6.9 ± 0.7 (3)

The Effects of PLA_2 Inhibition on Neutrophil Chemotaxis

Two aspects of chemotaxis were investigated, cell polarisation and orientated migration under agarose (Shields and Haston 1985, Nelson et al 1976). Neutrophil polarisation was stimulated dose dependently by LTB_4 and FMLP. Maximum polarisation was achieved by both stimulants at concentrations of 1×10^{-8}M (Fig 30). Neutrophils were then preincubated for 15 mins with PLA_2 inhibitors and stimulated with FMLP or LTB_4 at their optimum concentration. Lipocortin (1×10^{-6}M) and pBPB (5×10^{-6}M) had no effect on FMLP (1×10^{-8}M) stimulated polarisation (Table 6). Mepacrine (5×10^{-5}M) on the other hand inhibited the polarising response by 78.8%. Cells preincubated with cytochalasin B (5 ug/ml) (an agent known to inhibit chemotaxis by interacting with the cytoskeleton) also exhibited a reduced polarising response (77% inhibition). Likewise, the same concentrations of lipocortin did not inhibit the polarising response to LTB_4 (1×10^{-8}M), whilst mepacrine reduced polarisation by 87.1% (Table 6).

FMLP stimulated neutrophil migration dose dependently between 5×10^{-8} and 5×10^{-7}M (Fig 31). In three experiments neither lipocortin (5×10^{-6}M) nor pBPB (5×10^{-6}M) significantly inhibited orientated migration (Table 7).

Figure 30 - NEUTROPHIL POLARISATION STIMULATED BY LTB_4 AND FMLP .

Human neutrophils were incubated with FMLP and LTB_4 for 30 minutes and then fixed with 2.5% glutaraldehyde. Results are expressed as the percent of cells polarised in a sample count.

Each point prepresents the mean ± of three experiments.

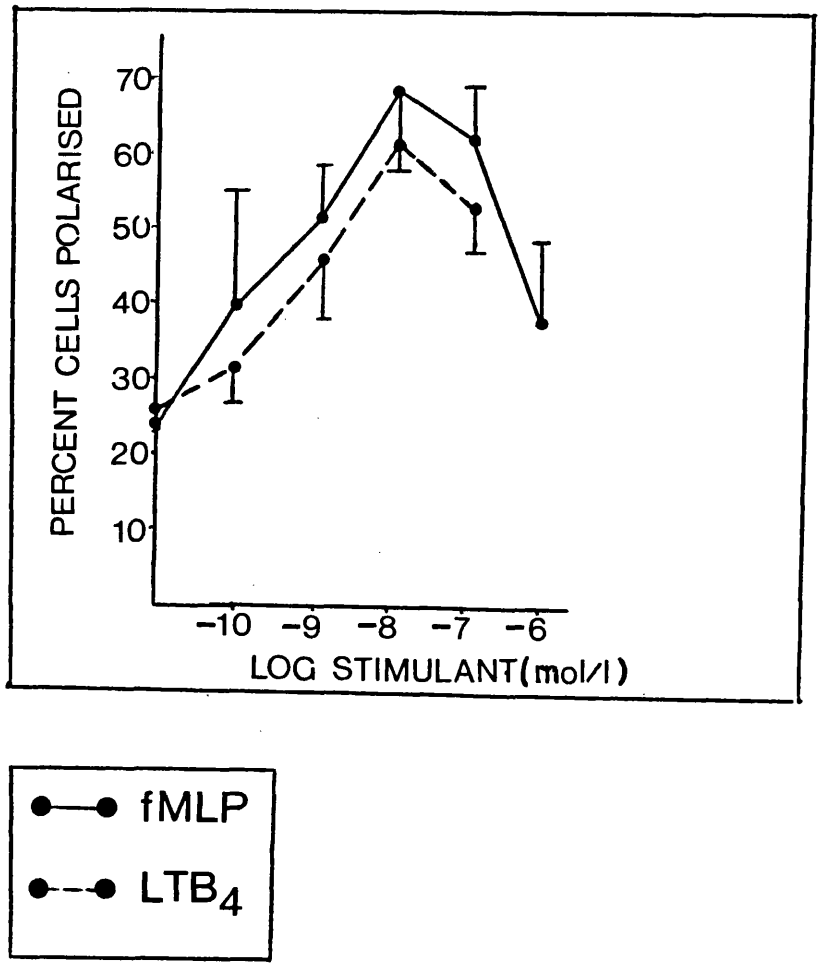


Table 6

The effect of PLA_2 inhibitors and cytochalasin B on the polarising response of human neutrophils to FMLP ($1 \times 10^{-8}M$) and LTB_4 (1×10^{-8}). Values indicate number of cells polarised compared to cells preincubated without inhibitor (100%).

*Subsequent investigation revealed that the batch of lipocortin employed had a very high endotoxin content.

	% OF CONTROL POLARISING RESPONSE	
INHIBITOR	FMLP ($1 \times 10^{-8}M$)	LTB_4 ($1 \times 10^{-8}M$)
CONTROL	100	100
LIPOCORTIN ($1 \times 10^{-6}M$)	103.0 (2)	306.0* (2)
pBPB ($5 \times 10^{-6}M$)	97.8 (2)	Not done
MEPACRINE ($5 \times 10^{-5}M$)	21.2 (2)	12.9 (2)
CYTOCHALASIN B (5 ug/ml)	22.3 (2)	18.3 (2)

Figure 31 - NEUTROPHIL CHEMOTAXIS STIMULATED BY FMLP.

Results are expressed as the ratio of stimulated migration towards FMLP/spontaneous migration towards buffer (ie the chemotactic index).

Each bar represents the mean of 3 experiments. (±SD)

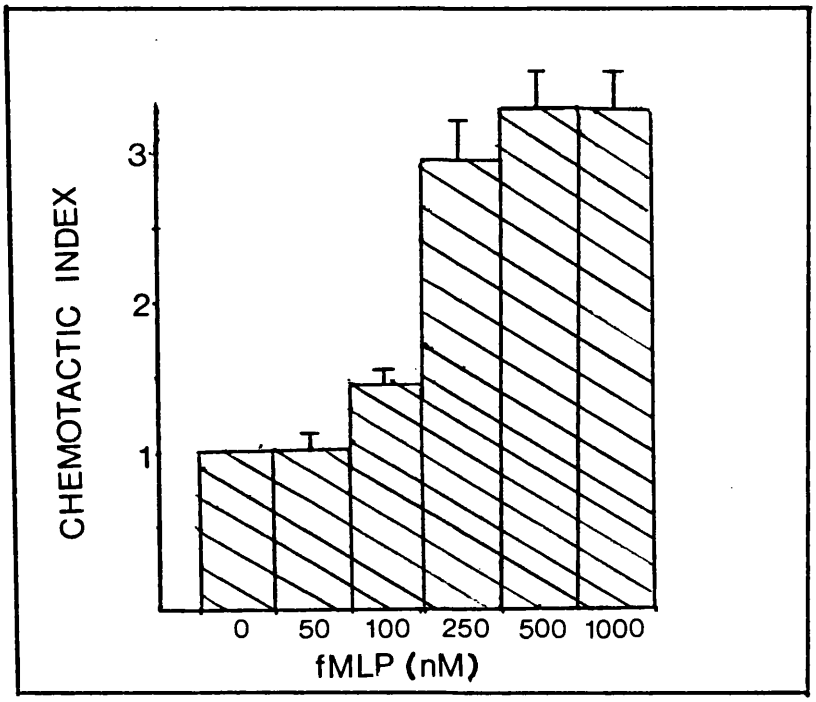


Table 7

The effect of phospholipase A_2 inhibitors on the chemotactic response of neutrophils stimulated by FMLP (1×10^{-6}M).

INHIBITOR	CHEMOTACTIC INDEX MEAN ± SD
NONE	3.5 ± 0.6
LIPOCORTIN (5×10^{-6}M)	3.6 ± 0.3
pBPB (5×10^{-6}M)	3.5 ± 0.9

CHAPTER 6

Neutrophil Activity in Connective Tissue Diseases and its Relation to Microvascular Damage

Oxidants generated by neutrophils *in vitro* are capable of mediating endothelial cell cytotoxicity and may play an important role in the microvascular damage associated with some connective tissue diseases (eg systemic sclerosis) (Blake *et al* 1984). Accordingly, basal and stimulated H_2O_2 production were measured in neutrophils from connective tissue disease patients where vascular injury forms an important component (ie systemic sclerosis, systemic lupus erythematosus and their overlapping variant, mixed connective tissue disease). In addition, for each patient group studied, serum levels of Factor VIII related antigen (FVIII Rag), were measured as an index of microvascular injury. The intention was to determine whether a relationship existed between neutrophil oxidative activity and the level of microvascular damage ie whether neutrophils were contributing to the vascular lesion.

Patient Groups

The patients selected for each disease group fulfilled their respective diagnostic criteria as defined by the Arthritis and Rheumatism Association (ARA 1980). Patients were classified as mixed connective tissue disease when they presented symptoms overlapping SS and SLE and were seropositive for the U_1RNP antibody (Sharp *et al* 1972).

Table 8

Summary of the details of the main patient groups with regard to number, age and disease duration.

GROUP	(n)	AGE (YRS) (MEAN ± SD)	DURATION (YRS)
CONTROL	26	38 ± 20	------
SS	34	56 ± 15	17 ± 10
MCTD	9	48 ± 15	14 ± 7
SLE	19	52 ± 20	8 ± 5
PR	7	40 ± 12	-------

Disease activity was defined for the SLE group by the presence of the following symptoms:-

Arthritis

Rash

Pleurisy

Nephritis

Neurological disease

Unexpected Fever

Weight Loss

Patients presenting with none of the listed symptoms were defined as inactive; with 1 symptom, mildy active and with 2 or more symptoms, active.

Drug Treatments

Patients in the SS group were generally taking medication for treatment of Raynaud's phenomenon. A small number were also being prescribed D-Penicillamine and colchicine. SLE patients were receiving low dose steroids (under 10 mg/day).

Hydrogen Peroxide Production by Neutrophils from Patients with Connective Tissue Diseases

In all the patient groups studied basal H_2O_2 production was significantly higher than the healthy control group (Fig 32). A similar pattern of responses was observed following stimulation with aggregated IgG with SS, MCTD and SLE patient groups generating significantly more H_2O_2 than controls (Fig 33). In contrast H_2O_2 production by the primary Raynaud's group did not differ significantly from control. When FMLP was employed as the stimulus only the MCTD group generated significantly more H_2O_2 than control ($p< 0.001$) (Fig 34). Similar results for the SS group have been reported from this laboratory by Maslen et al (1987). Tables 9 - 11 summarise the data collected from these studies. All groups generated similar levels of H_2O_2 in response to PMA (Fig 35).

When the SS group was subdivided by the extent of skin involvement H_2O_2 production stimulated by FMLP and IgG was significantly higher than control in the sclerodactyly group (Fig 36) ($p< 0.05$). Neutrophils from patients with more diffuse skin involvement did not generate significantly more H_2O_2 following stimulation than control. Thus, with regards to

FMLP and IgG stimulated neutrophil activity, a clear distinction between two variants of the same disease can be observed.

Basal H_2O_2 production was significantly higher than control in both SS subgroups. When the SS group was subdivided on the basis of autoantibody profiles no significant differences were found in the anticentromere -/+ or scl 70 -/+ groups (Table 12). The clinical manifestations of SS would therefore seem to be a more important determinant of neutrophil activity than serological profiles.

Basal H_2O_2 production in the SLE group was significantly higher than control ($p< 0.001$). The elevated levels of H_2O_2 production in this group were not related to the disease activity scores of the patients or any drug treatments (data not shown). When basal H_2O_2 production was related to the patients autoantibody profiles no significant differences were found between SLE patients seropositive or seronegative for anti DNA or anti U_1RNP autoantibodies (Table 13). However, mean basal H_2O_2 production values in both SLE seropositive groups were higher than patients without the respective autoantibodies. Therefore further studies in this area to increase patient numbers in each group may well reveal significant differences.

Figure 32 - BASAL HYDROGEN PEROXIDE PRODUCTION BY NEUTROPHILS FROM PATIENTS WITH AND WITHOUT CONNECTIVE TISSUE DISEASE

Human neutrophils were added to H_2O_2 assay mixture containing PBSG (to measure unstimulated H_2O_2 production). H_2O_2 production expressed as umol/l/10^6 cells/30 minutes. Each point represents the mean of two determinations for each patient.

Horizontal bars indicate meadian values for each subject group.

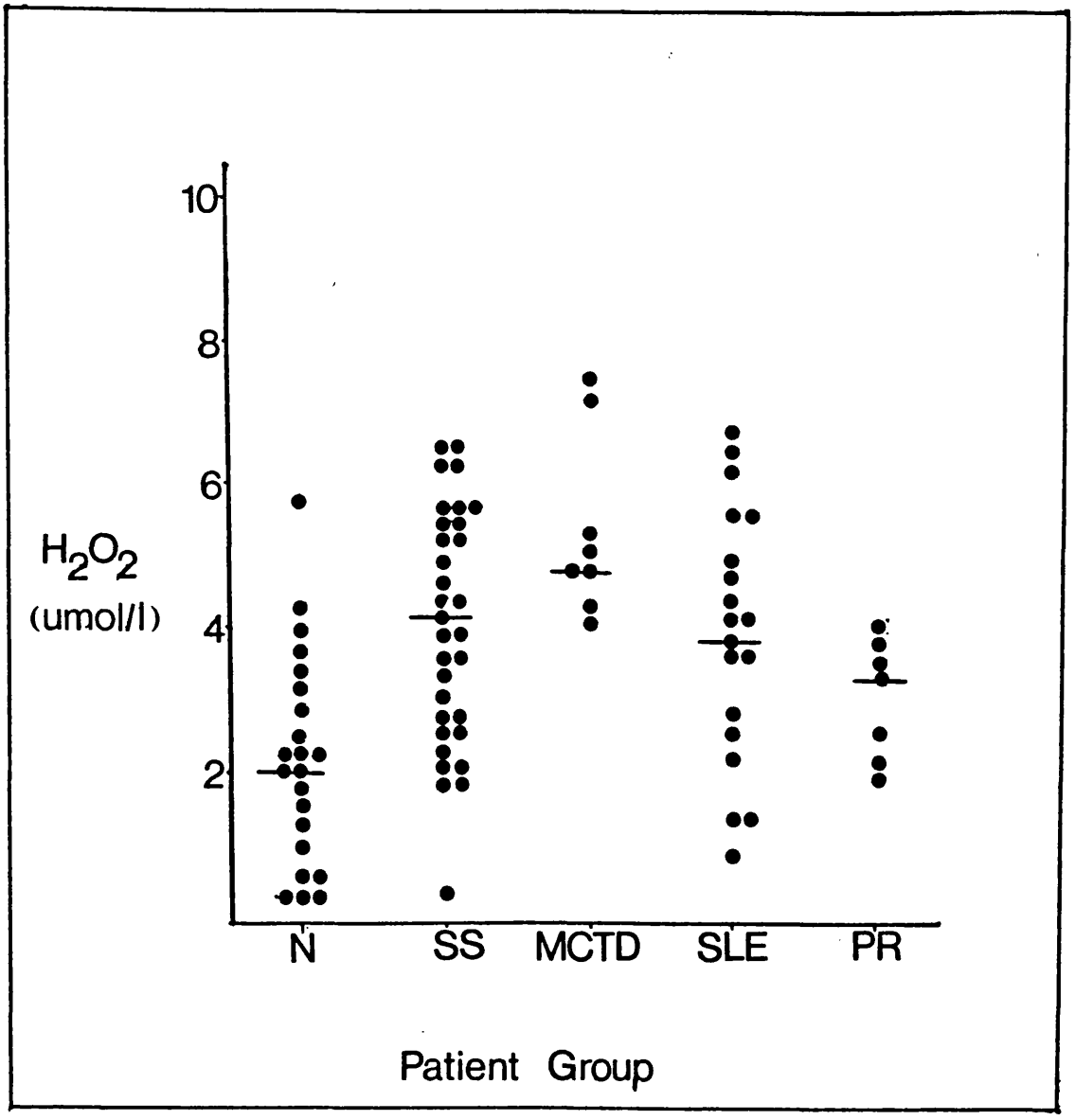


Table 9

Data summary for basal hydrogen peroxide production by subjects with or without connective tissue disease.

Unstimulated (Basal) H_2O_2 Production (umol/l)				
Group	n	median	mean±sd	p value
CONTROL	26	2.1	2.3 ± 1.4	---
SS	34	4.2	4.2 ± 1.7	< 0.001
MCTD	9	5.4	5.6 ± 1.1	< 0.001
SLE	19	4.2	4.2 ± 1.6	< 0.001
PR	7	3.4	3.7 ± 1.5	< 0.01

Figure 33 - HYDROGEN PEROXIDE PRODUCTION STIMULATED BY AGGREGATED IgG IN NEUTROPHILS FROM SUBJECTS WITH AND WITHOUT CONNECTIVE TISSUE DISEASE

Human neutrophils were added to H_2O_2 assay mixture containing either aggregated IgG (200 ug/ml) or PBSG (to measure unstimulated H_2O_2 production). H_2O_2 production expressed as umol/l/10^6 cells/30 minutes.

Each point represents the mean of two determinations for each patient.

All values adjusted for resting H_2O_2 production.

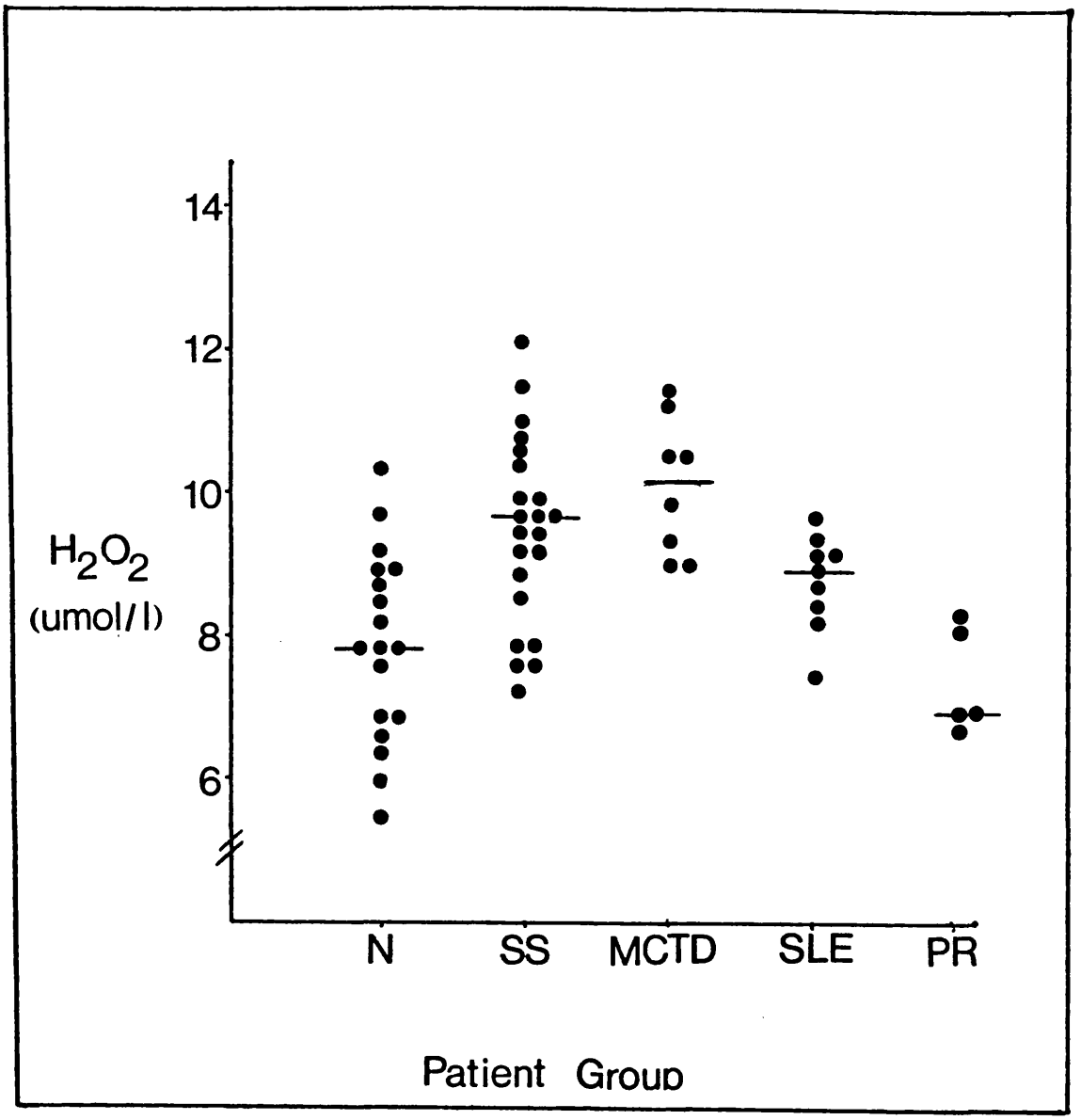


Table10

Data summary for aggregated IgG stimulated hydrogen peroxide production in subjects with and without connective tissue disease.

Aggregated IgG (200ug/ml) stimulated H_2O_2 (umol/l)				
Group	n	median	mean±sd	p value
CONTROL	18	7.6	7.6 ± 1.3	
SS	22	8.9	8.9 ± 1.2	< 0.05
MCTD	8	9.7	10.3 ± 0.9	< 0.01
SLE	9	8.5	8.4 ± 1.0	NS
PR	5	7.8	7.6 ± 1.7	NS

Figure 34 - HYDROGEN PEROXIDE PRODUCTION STIMULATED BY FMLP IN NEUTROPHILS FROM SUBJECTS WITH AND WITHOUT CONNECTIVE TISSUE DISEASE

Human neutrophils were added to H_2O_2 assay mixture containing either FMLP ($5 \times 10^{-6}M$) or PBSG (to measure unstimulated H_2O_2 production). H_2O_2 production expressed as umol/l/10^6 cells/30 minutes.

Each point represents the mean of two determinations for each patient.

All values adjusted for resting H_2O_2 production.

Horizontal bars indicate meadian values for each subject group.

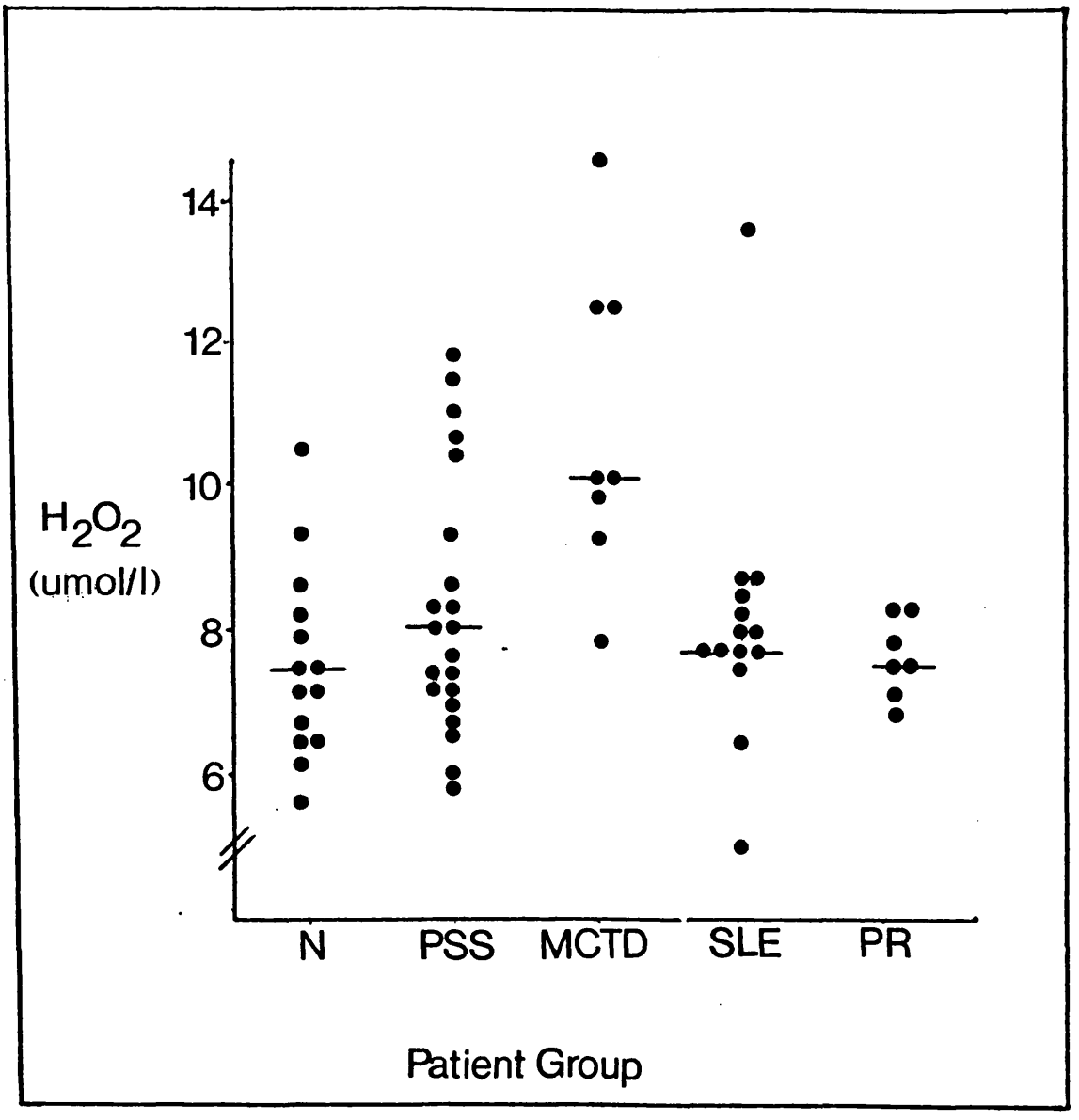


Table 11

Data summary for FMLP stimulated hydrogen peroxide prodcution in subjects with and without connective tissue disease.

fMLP(5×10^{-6}M) stimulated H_2O_2 (umol/l)				
Group	n	median	mean±sd	p value
CONTROL	14	7.6	7.4 ± 1.4	
SS	21	3.1	8.9 ± 2.0	NS
MCTD	8	10.0	10.8 ± 2.1	<0.01
SLE	14	7.8	8.1 ± 1.8	NS
PR	7	7.7	7.8 ± 1.2	NS

Figure 35 - HYDROGEN PEROXIDE PRODUCTION STIMULATED BY PHORBOL MYRISTRATE ACETATE IN NEUTROPHILS FROM SUBJECTS WITH AND WITHOUT CONNECTIVE TISSUE DISEASE

Human neutrophils were added to H_2O_2 assay mixture containing either PMA (10 ng/ml) or PBSG (to measure unstimulated H_2O_2 production). H_2O_2 production expressed as umol/l/10^6 cells/30 minutes.

Each point represents the mean of two determinations for each patient.

All values adjusted for resting H_2O_2 production.

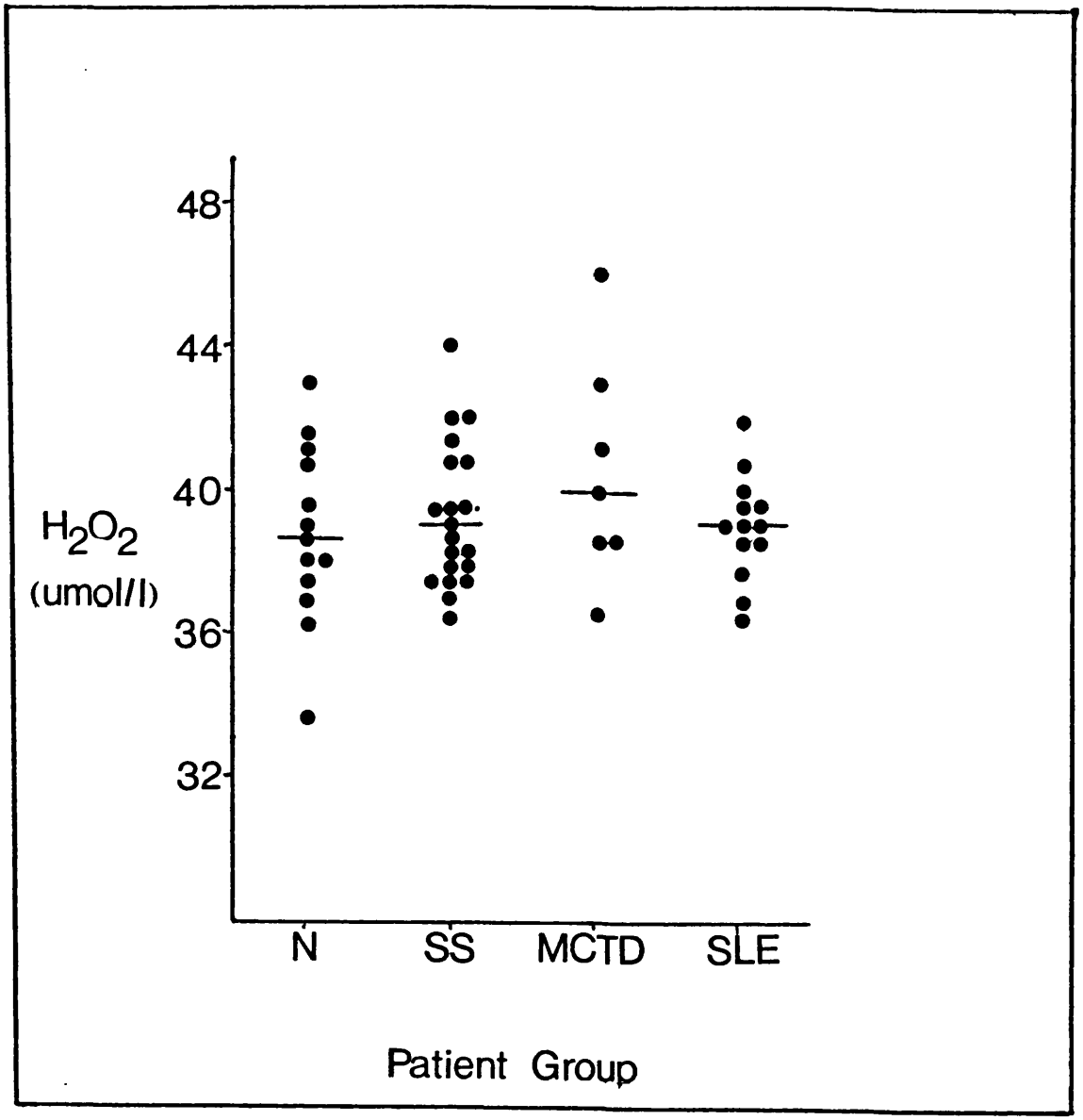
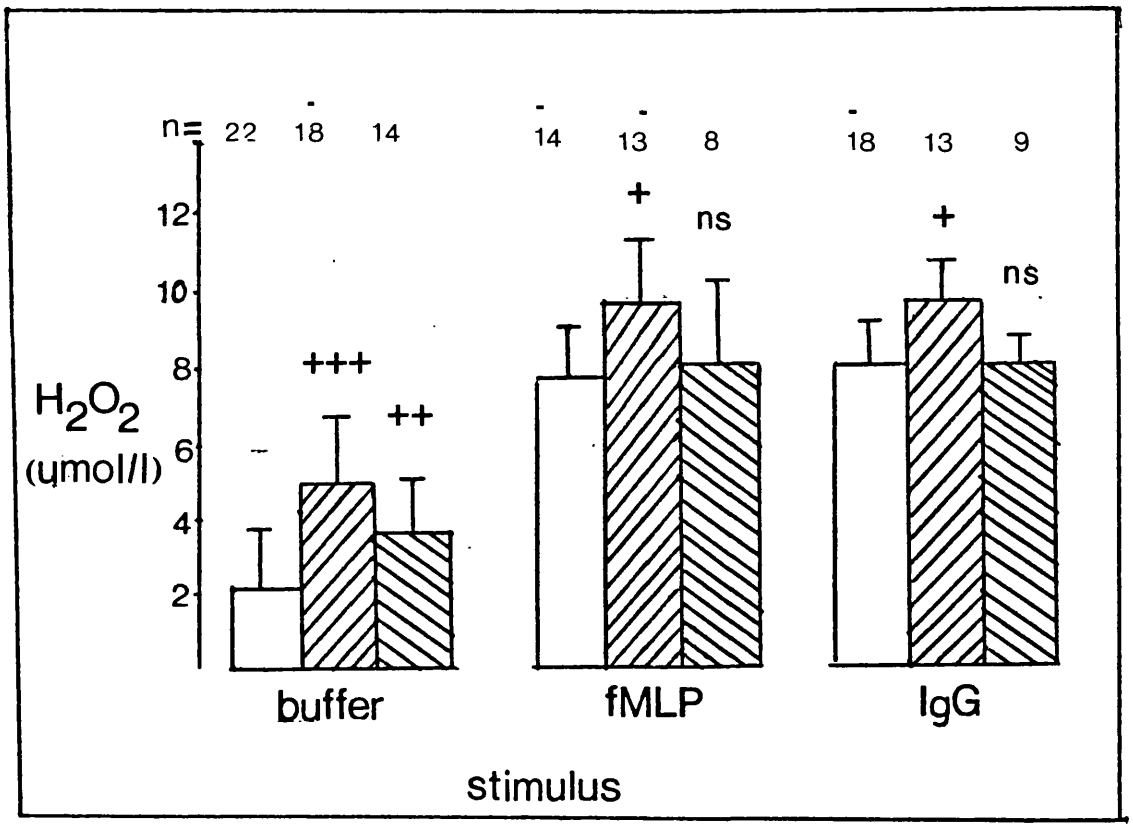


Figure 36 - BASAL AND STIMULATED HYDROGEN PEROXIDE PRODUCTION IN SUBGROUPS OF SYSTEMIC SCLEROSIS AND HEALTHY CONTROL SUBJECTS

H_2O_2 production expressed in umol/l/10^6 cells/30 minutes.



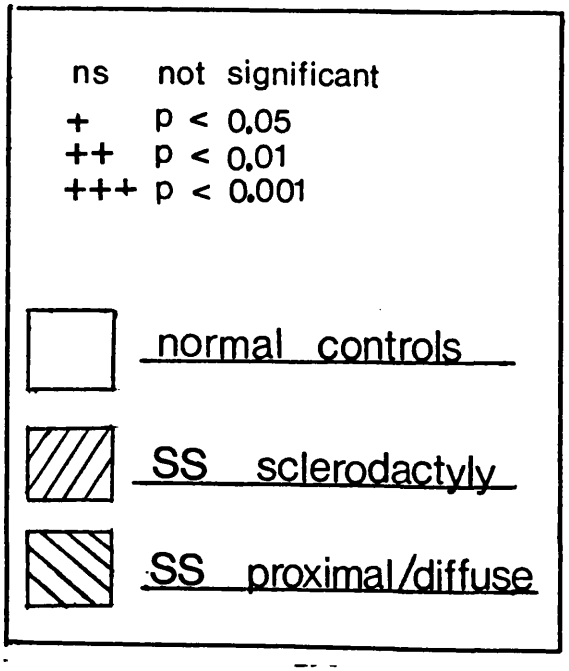


TABLE 12

Unstimulated hydrogen peroxide production in SS patients subclassified by their autoantibody profiles.

H_2O_2 - unstimulated (umol/l)			p
ANA	— MEAN ± SD	+ MEAN ± SD	value
Anti-CENTROMERE	3.8 ± 1.9 (17)	4.2 ± 1.7 (9)	ns
Anti-Scl 70	4·1 ± 2·4 (4)	4·3 ± 1·4 (21)	ns

TABLE 13

Unstimulated hydrogen peroxide production in SLE patients subclassified by their autoantibody profiles.

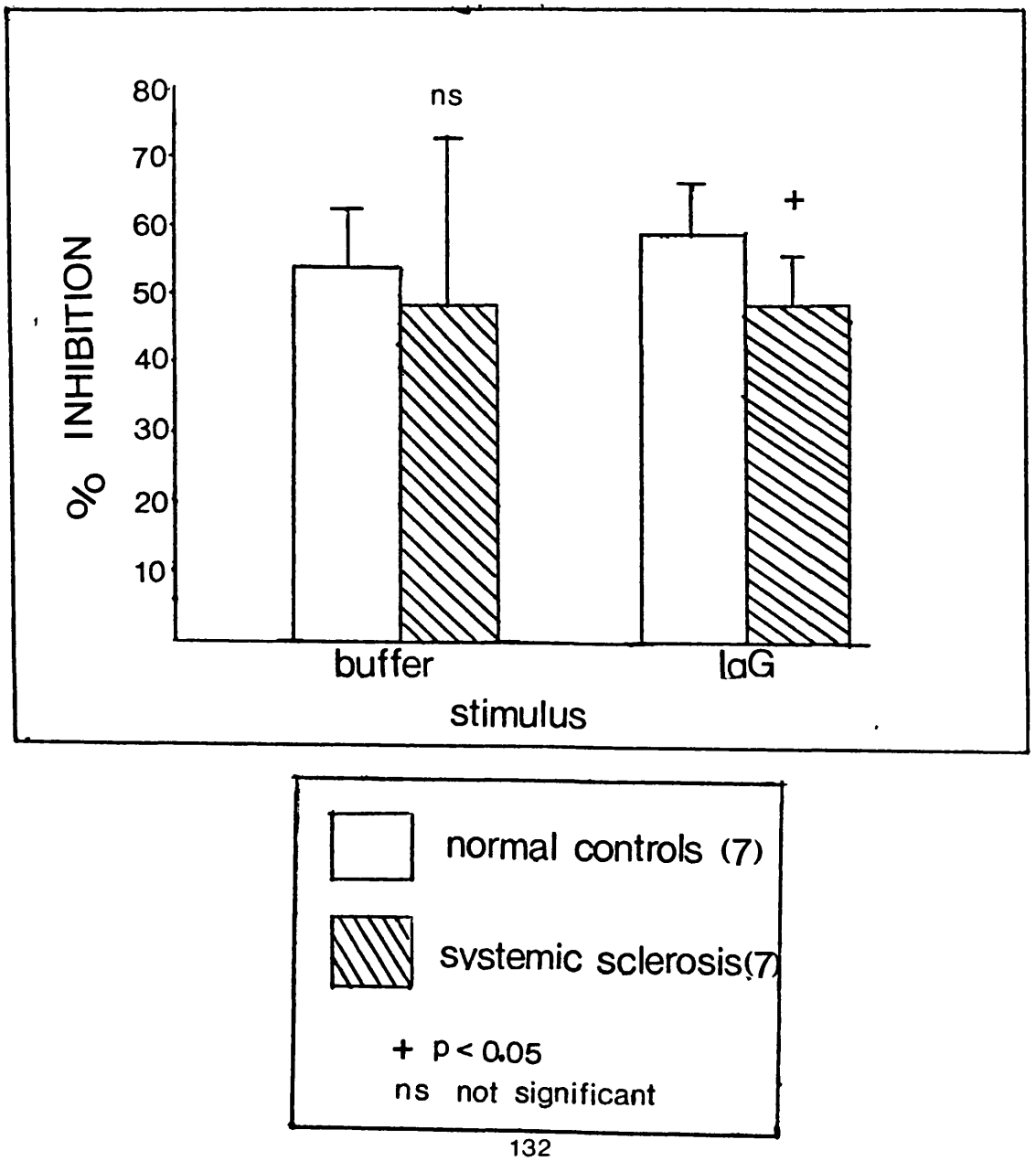
H_2O_2 - unstimulated (umol/l)			p
ANA	— MEAN ± SD	+ MEAN ± SD	value
Anti-DNA	3.0 ± 1.2 (4)	4.1 ± 1.7 (9)	ns
Anti-U_1RNP	4.1 ± 1.5 (13)	4.6 ± 2.0 (5)	ns

H_2O_2 Production in SS: Sensitivity to cAMP

Several workers have reported a defective stimulated cAMP response in cells from patients with systemic sclerosis (Kirby et al 1980, Belch et al 1985). Accordingly the effects of salbutamol, an agent which elevates cAMP by stimulating B_2 adenergic receptors, on basal and IgG stimulated H_2O_2 production were investigated (Fig 37). In response to stimulation with IgG the inhibition of H_2O_2 production by salbutamol was significantly less in the SS patients than healthy control subjects. ($p< 0.05$). No significant differences in the effects of salbutamol on basal H_2O_2 production were observed between the two groups.

Figure 37 - THE DIFFERENTIAL EFFECTS OF SALBUTAMOL ON BASAL AND IgG STIMULATED HYDROGEN PEROXIDE PRODUCTION IN HEALTHY CONTROL SUBJECTS AND PATIENTS WITH SS

Neutrophils were incubated in the H_2O_2 assay mixture containing salbutamol (1.5×10^{-4}M) amd ether buffer (PBSG) or aggregated IgG (200 ug/ml). Results are expressed as percent inhibition of H_2O_2 produced by resting and stimulated cells in the absence of salbutamol.



Myeloperoxidase Release in SS

In order to determine whether enhanced neutrophil activity in the SS group was limited to oxidative metabolism, basal and IgG stimulated myeloperoxidase release were measured in 4 SS patients.

A concentration response curve was first constructed to find the optimum concentration of IgG to be used in the patient study. Aggregated IgG enhanced MPO release dose dependently with maximum release occurring at a concentration of 200 ug/ml (Fig 38). In the patient studies 4 subjects with SS were compared with 4 matched controls. Unstimulated MPO release from SS patients was significantly higher than control ($p < 0.05$ (Fig 39). Total IgG stimulated MPO content of the cells did not vary significantly between the two groups.

Figure 38 - MYELOPEROXIDASE RELEASE BY HUMAN NEUTROPHILS STIMULATED BY AGGREGATED IgG

Human neutrophils were incubated with either buffer (PBSG) aggregated IgG or triton x100. Basal and stimulated MPO release is expressed as a percent of total enzyme release (ie that caused by triton).

Bars represent mean ± standard deviation of 3 experiments.

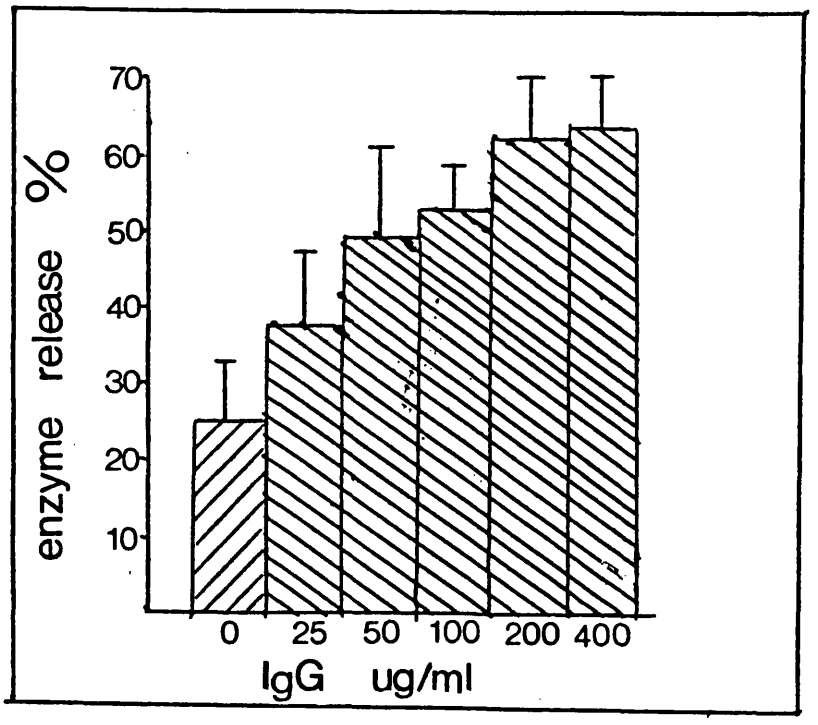
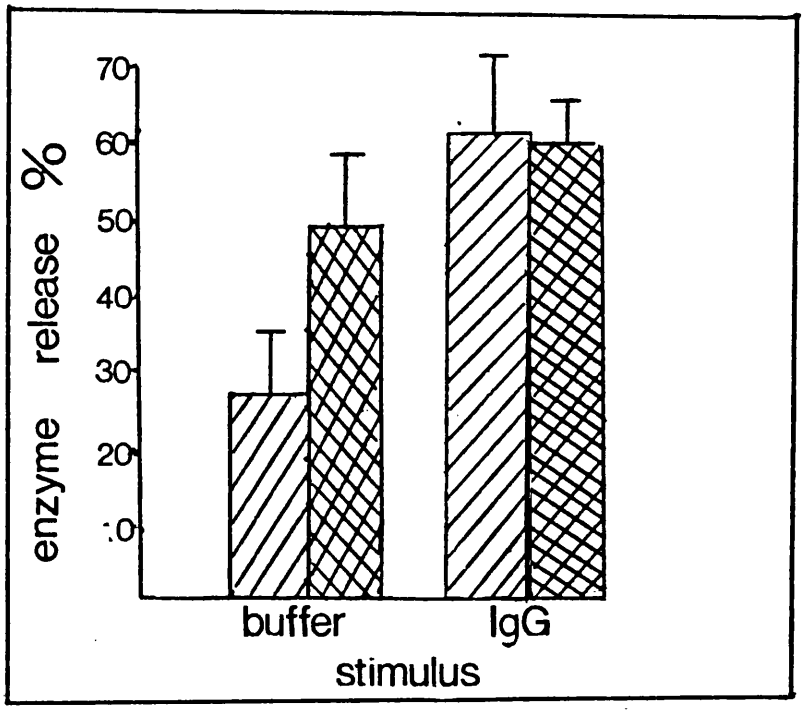
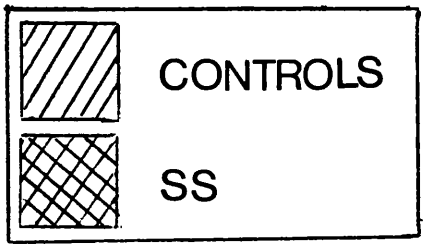


Figure 39 - BASAL AND AGGREGATED IgG STIMULATED MYELOPEROXIDASE RELEASE IN HEALTHY CONTROL SUBJECTS AND PATIENTS WITH SYSTEMIC SCLEROSIS

Healthy or SS patient neutrophils were incubated with either buffer (PBSG) aggregated IgG or triton x100. Basal and stimulated MPO release is expressed as a percent of total enzyme release (ie that caused by triton).

Bars represent mean ± standard deviation of 3 experiments.





Factor VIII Rag Levels in Connective Tissue Disease

Products released from stimulated neutrophils have been implicated as mediators of microvascular injury. In a retrospective study levels of factor VIII related antigen (a serological marker of vascular damage) levels were measured by ELISA in sera from patients with SS, SLE, MCTD and primary Raynauds Disease (Fig 41). Absolute levels were determined using a standard curve (Fig 40) constructed from pooled healthy human serum. A linear relationship between absorbance and of the standard dilution between 1/10 and 1/320 were observed.

In patient studies, Factor VIII Rag levels were determined from a 1/40 serum dilution. Elevated levels were initially found in SS, MCTD and SLE groups ($p < 0.001$) (Fig 41, Table 14) but not in the primary Raynauds group. In a subsequent study however, employing larger numbers of Raynauds patients levels were elevated (Fig 42). Furthermore, elevated F VIII Rag levels were found in sera from patients with silica induced systemic sclerosis. For each patient in the SS group Factor VIII Rag levels were then correlated with basal neutrophil activity (Fig 43). No significant correlation was observed ($r = 0.25$ NS) . However, it is important to note that serum samples for the factor VIII studies were not taken at the same times as blood for the neutrophils and further studies utilising data from the same blood samples are warranted. Longitudinal studies over a nine month period revealed that basal and stimulated hydrogen peroxide production in one SS patient and one healthy subject were constant. Whether F VIII Rag levels follow a similar pattern has yet to be determined.

Elevated factor VIII Rag levels in the SS group did not correlate with serological profiles but occurred in a higher incidence in patients with fibrosis limited to sclerodactyly than those with more diffuse skin involvement.

Figure 40 - CURVE FOR STANDARDISING THE F VIII Rag ASSAY

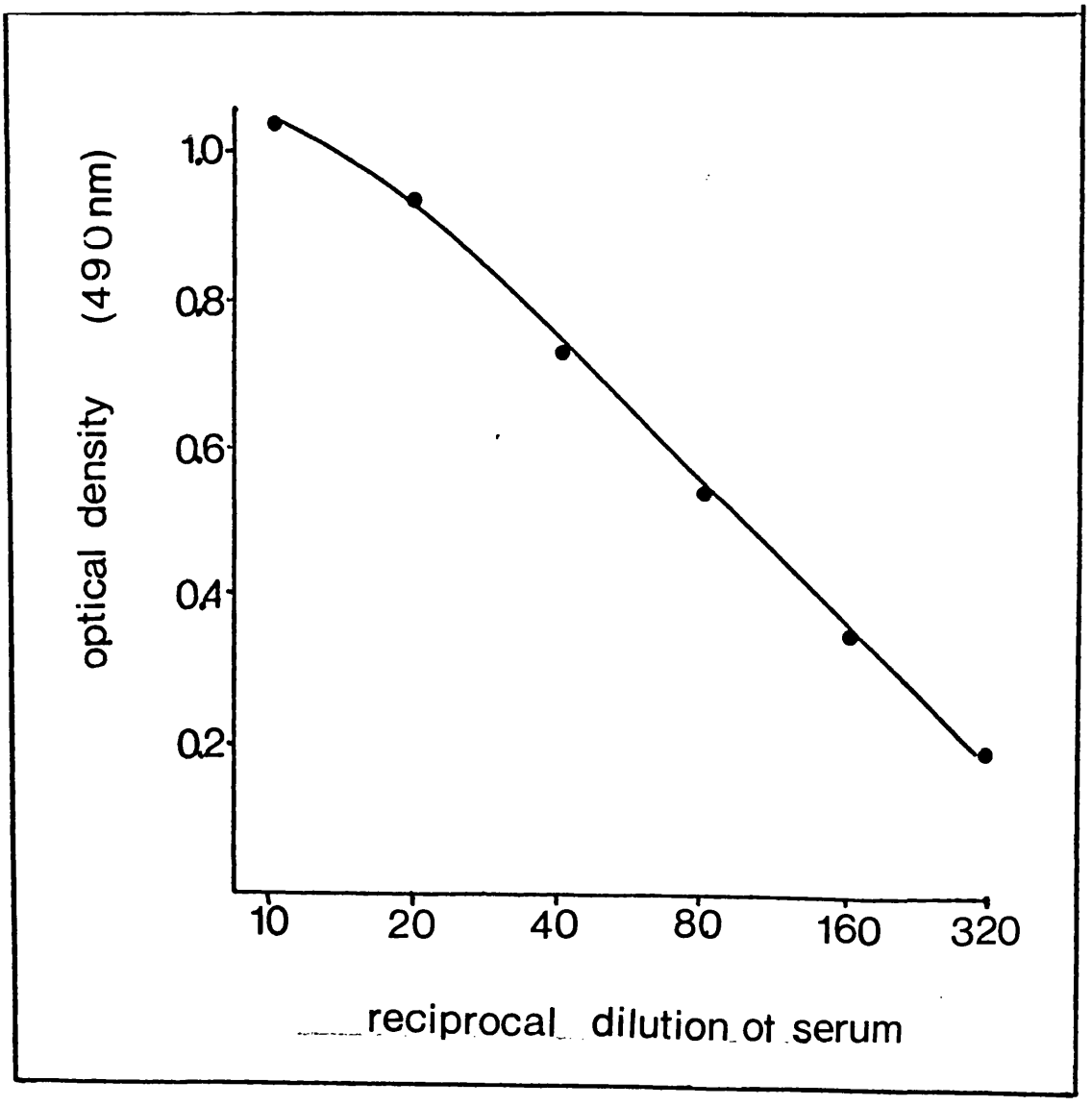
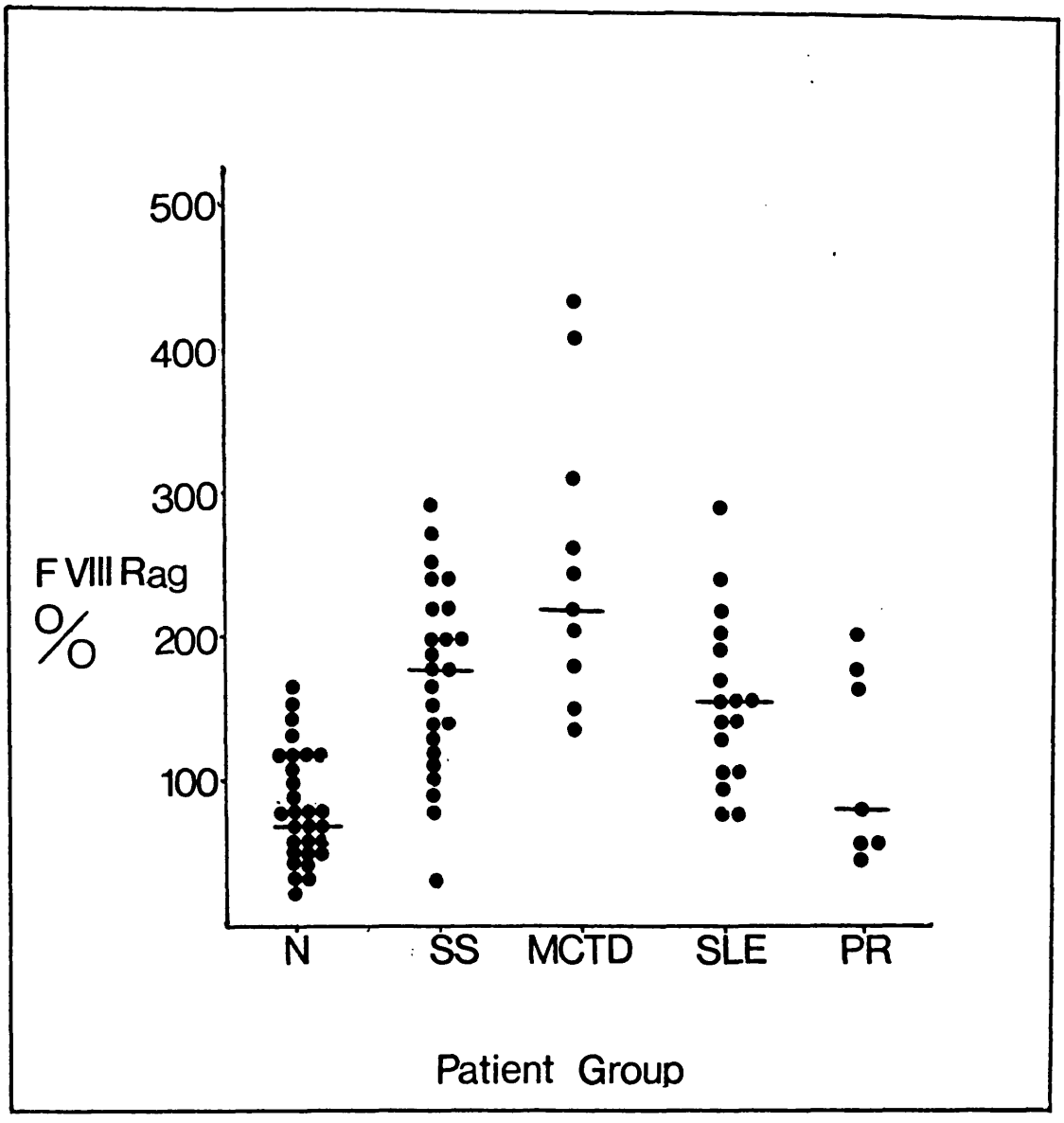


Figure 41 -SERUM F VIII Rag LEVELS IN SUBJECTS WITH AND WITHOUT CONNECTIVE TISSUE DISEASE

F VIII Rag levels in subjects are expressed as a percent of a pooled normal human serum standard.

Points represent the mean of two determinations per patient.



Horizontal bars indicate meadian values for each subject group.

Table 14

Data summary for serum FVIII Rag levels in patients with or without connective tissue disease.

Factor VIII Related Antigen %				
GROUP	n	median	mean±sd	pvalue
CONTROL	30	80	86±30	---
SS	29	188	179±75	< 0.001
MCTD	10	200	234±102	< 0.001
SLE	18	147	153±53	< 0.001
PR	9	144	144±112	NS

Figure 42 - SERUM F VII Rag LEVELS IN NORMAL SUBJECTS AND PATIENTS WITH PRIMARY RAYNAUD'S DISEASE AND SILICA INDUCED SYSTEMIC SLCEROSIS

F VIII Rag levels in subjects are expressed as a percent of a pooled normal human serum standard.

Points represent the mean of two determinations per patient.

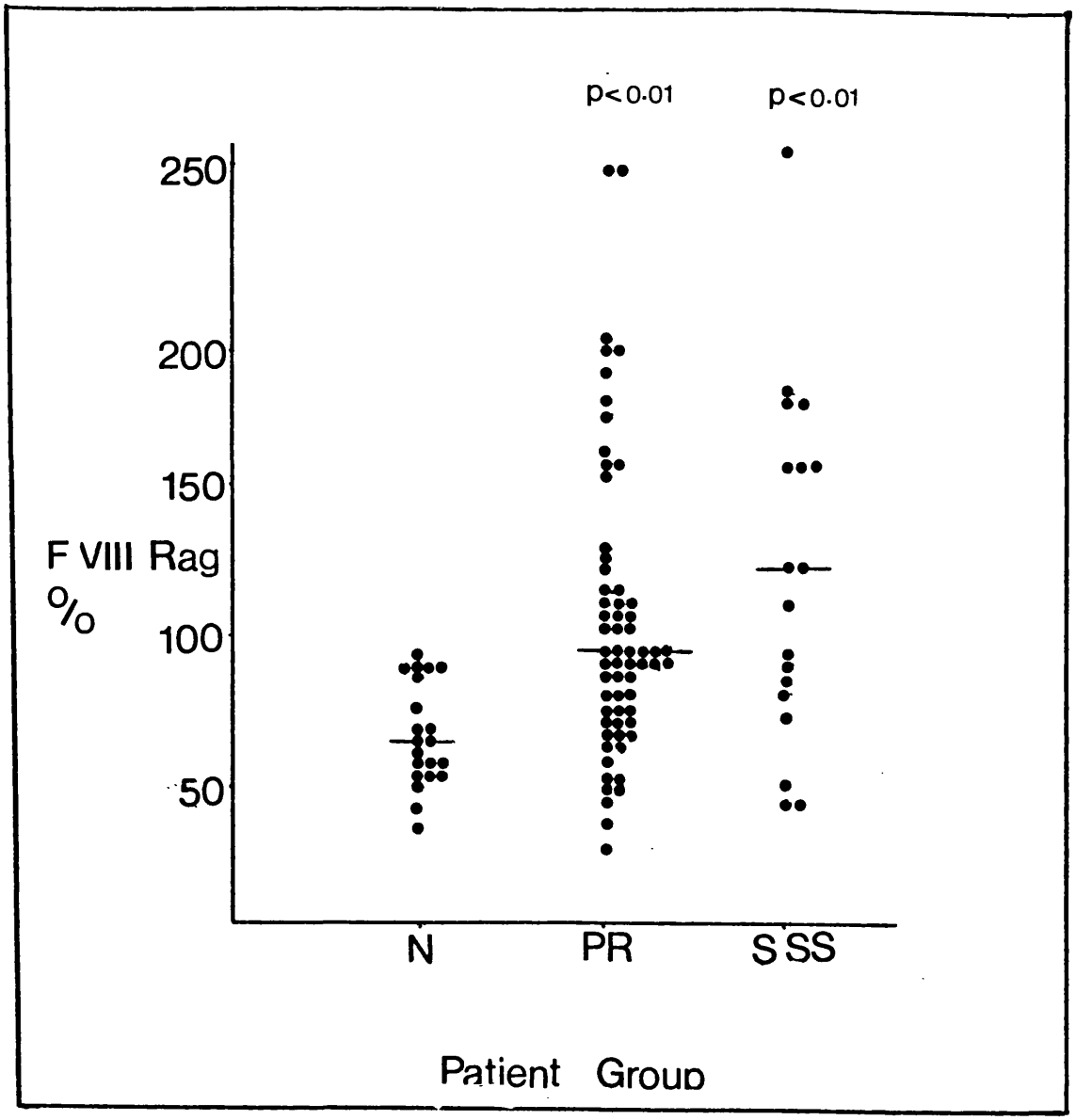
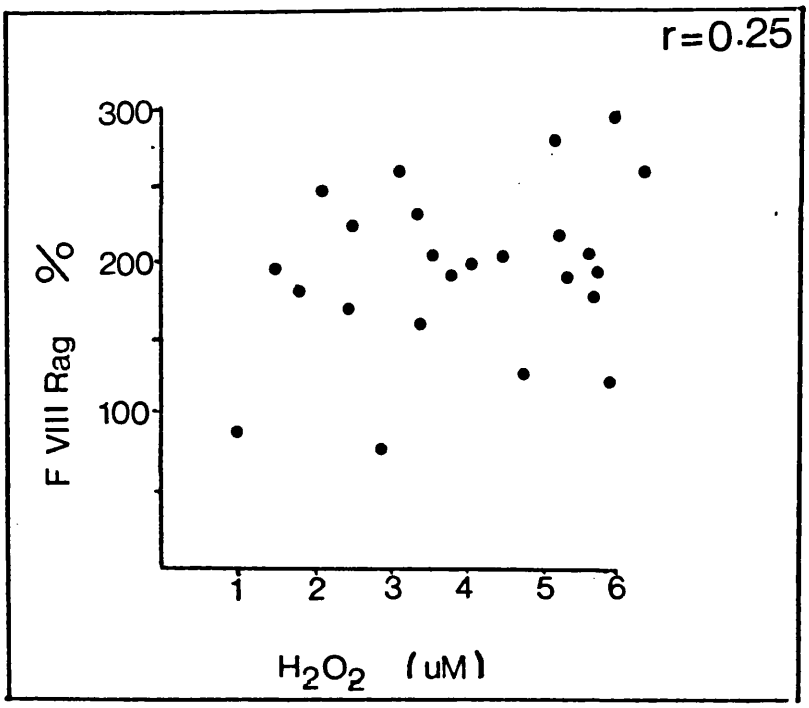


Figure 43 - CORRELATION BETWEEN SERUM F VIII Rag AND BASAL HYDROGEN PEROXIDE PRODUCTION IN PATIENTS WITH SYSTEMIC SCLEROSIS



CHAPTER 7

Discussion

Having established using diphenylene iodonium that basal and stimulated hydrogen peroxide was derived from NADPH-oxidase, the molecular mechanisms involved in activating the enzyme were investigated. Two groups of agents known to inhibit neutrophil activity were employed: the first, included agents which elevate intracellular cAMP whilst the second group consisted of agents whose primary action is that of inhibiting phospholipase A_2.

The aim of these studies was to highlight and differentiate the mechanism of NADPH oxidase activation by different stimuli. In addition, the effect of PLA_2 inhibition was also investigated on the chemotactic and polarising responses of neutrophils to determine whether the molecular mechanisms of cell activation were specific not only for a particular stimulant but also for a given response to that stimulant.

The final section of this discussion deals with the role of neutrophil derived oxidants in the pathophysiology of microvascular damage in connective tissue diseases and in particular in the pathogenesis of systemic sclerosis

The Effects of Agents Which Increase cAMP on Basal and Stimulated H_2O_2 Production.

Since its discovery by Sutherland in the late 1950's cyclic adenosine monophosphate (cAMP) has been implicated in many aspects of cell activity

(Gancedo et al 1985). Intracellular cAMP levels are regulated by two enzymes: adenylate cyclase and cAMP phosphodiesterase. The former catalyses cAMP synthesis from adenosine triphosphate whilst the later degrades it to adenosine monophosphate (Walsh et al 1968). In general, the effects of cyclic AMP are mediated through cAMP dependent phosphorylating (kinase) enzymes (Gancedo et al 1985). Activation of these involves the removal of inhibitory subunits from the enzymes catalytic site. cAMP dependent kinases can regulate the activity of specific enzymes either by enhancing their catalytic activity or their sensitivity to substrates. Alternatively, they can alter the function of non enzymic proteins which control important cellular processes (eg cell division and changes in membrane permeability (Cohen 1985)).

A rise in intracellular cAMP levels in neutrophils generally leads to the inhibition of stimulated cellular activity (Ignarro and George 1974). An interesting phenomenon, however, is the small transient increase in cAMP levels that accompanies neutrophil stimulation (Marigancello et al 1971). Temporally this lags behind membrane hyperpolarisation but preceeds the functional response. It can, however, occur independently of both of these (Smolen et al 1980). The function of this apparently innocuous rise in cAMP is unknown, but it appears to occur by a different pathway from G_s, and requires the participation of calcium and calmodulin (Verghese et al 1985).

In this thesis, the effects of four agents that stimulate a rise in intracellular cAMP have been investigated on basal and stimulated H_2O_2 production. These included adrenaline, an adrenomedullary hormone released into the

circulation during periods of stress; the selective β_2 adrenoceptor agonist salbutamol; adenosine, a naturally occurring purine intermediate and Iloprost, a synthetic prostacyclin analogue. Adrenaline exerts a bidirectional control over adenylate cyclase activity. Stimulation of B_2 adrenergic receptors by adrenaline leads to activation of the enzyme through Gs and a rise in intracellular cAMP whilst α_2 receptor stimulation inhibits the Gs mediated activation of adenylate cyclase. Adenosine has been reported to activate adenylate cyclase by stimulating purinergic A_2 receptors (Schwabe 1982). Engler (1987) has recently hypothesized that the inhibition of neutrophil activation by adenosine is an important physiologic mechanism for limiting myocardial reperfusion injury. Iloprost, like prostaglandins of the E and I series elevates cAMP by stimulating specific cell surface receptors. The rise in cAMP stimulated by prostaglandins has been reported to correlate well with their capacity to inhibit neutrophil superoxide production (Fantone and Kinnes 1983).

The inhibitory effects of adrenaline and salbutamol on H_2O_2 production were not stimulus specific. Direct antioxidant activity was apparent with both agents but contributed in only a minor way to the overall inhibitory response. The effects of yohimbine (a selective α_2 antagonist) demonstrated that stimulation of B_2 and α_2 receptors on neutrophils could modulate functional activity. The small increase in the inhibitory effects of adrenaline on FMLP stimulated H_2O_2 production by yohimbine indicated that although adrenaline was stimulating α_2 receptors its predominant effects were mediated by the B_2 receptor subtype. These observations agree well with the work of Panosian and Marinetti (1983). They reported that blockade of α_2 receptors on neutrophils with yohimbine

(at similar concentrations to those employed in this thesis) augmented the cAMP response to adrenaline. The effects of yohimbine on the salbutamol response were not investigated but it is unlikely that any inhibition by this agent would be augmented since its effects on adenylate cyclase are mediated entirely through B_2 receptor stimulation.

Salbutamol and adrenaline were most effective inhibitors of stimulated H_2O_2 production when incubated with cells together with the stimulant. Both agents were less effective at inhibiting basal H_2O_2 production. PMA, FMLP and aggregated IgG have all been reported to stimulate an apparently innocuous rise in intracellular cAMP in neutrophils. The rise in cAMP that accompanies neutrophil stimulation with PMA is probably due to the direct activation of adenylate cyclase through a phosphorylation step mediated by protein kinase C (Yoshimasa et al 1987). FMLP on the other hand is thought to elevate cAMP by stimulating adenylate cyclase indirectly through a Ca^{2+}/calmodulin dependent mechanism (Verghese et al 1985). In view of the preferential inhibition of stimulated over basal H_2O_2 production (which presumably is not accompanied by a rise in intracellular cAMP) it appears that the cAMP responses produced by adrenaline and the stimulants are synergistic; the overall result being to convert an innocuous rise in cAMP into one which inhibits cellular activity. Ignarro and George (1974) have reported augmented cAMP responses and inhibition of lysosomal enzyme release by adrenaline. In their study neutrophil cAMP levels were elevated four fold by adrenaline in the presence of aggregated IgG compared with buffer. When neutrophils were incubated for 5 minutes or longer with adrenaline prior to stimulation with FMLP the subsequent inhibition of H_2O_2 production was reduced. Agonist

induced rises in neutrophil cAMP are maximal by 3-5 minutes and then fall off rapidly (Smolen et al 1980). This may be due to the presence of a highly active phosphodiesterase enzyme in these cells (Weissmann 1987). On a background of cellular phosphatase activity and rapid reversal of cAMP dependent phosphorylation this may lead to poorer inhibition of cellular activity.

The apparent synergy of responses described above may account for the stimulus specific effects of Iloprost. This agent was ineffective at inhibiting responses to PMA and aggregated IgG. High concentrations of Iloprost produced only a small inhibition of basal H_2O_2 production, but effectively inhibited the FMLP response. These observations agree in part with those of Fantone and Kinnes (1983) who reported that high concentrations of another prostaglandin, PGE_1 ($4 \times 10^{-5}M$) were required to inhibit stimulated neutrophil superoxide production. Ishitoya and Takenawa (1987) have recently reported a calcium dependent synergistic rise in cAMP between FMLP and PGE_1. A similar co-operation may be occurring between Iloprost and FMLP. The ability of FMLP but not aggregated IgG or PMA to mobilise intracellular calcium may account for the stimulus specific synergism in cAMP response and subsequent inhibition of FMLP stimulated H_2O_2 production (Becker et al 1981). Another explanation for the stimulus specific effects of Iloprost may relate to the magnitude of the cAMP response. Certainly, the rise in cAMP that is stimulated by prostaglandins (eg PGE_1) is considerably lower than that by, for example B_2 agonists (Ignarro and George 1974, Ishitoya and Takenawa 1987). The small rise in cAMP that accompanies exposure to iloprost may lead to the inhibition of specific enzymes whilst larger increments

might result in gross structural changes. For example, hormonal stimulation of cyclic AMP has been reported to inhibit inositol phospholipid (PI) metabolism and the activity of phospholipid methyltransferases (Kato et al 1986, Kelly and Wong 1987). H_2O_2 production stimulated by FMLP is known to be at least in part dependent on PI breakdown (Takenawa et al 1985). This is certainly not the case for PMA stimulated H_2O_2 (Tauber 1987) production and in view of the data presented here may not be important for responses to aggregated IgG. Furthermore with regard to data to be discussed later in this thesis FMLP stimulated H_2O_2 production appears also to be dependent on phospholipid metabolism by the PLA_2 dependent pathway. Inhibition of phospholipid methyltransferase would inhibit the conversion of phosphatidylethanolomine to phosphatidylcholine and thereby inhibit PLA_2 by substrate depletion (Hirata et al 1979). Of course the larger increments in cyclic AMP that would occur with B agonists also affect the former reaction but in addition might have effects on the cell membrane and possibly NADPH oxidase itself. Such an action would therefore inhibit neutrophil H_2O_2 production regardless of the stimulant. Obviously a meaurement of absolute cAMP levels and enzyme activities would be required to substantiate this proposal.

The Effect of Phospholipase A_2 Inhibitors on Basal and Stimulated Neutrophil Activity

The molecular mechanisms leading to NADPH oxidase activation in human neutrophils are poorly defined. Modification to membrane phospholipids appears to be an important event for some stimulants. FMLP stimulated $O_2^{-\cdot}$ production, for example, is intimately linked to the activation of

phospholipase C and breakdown of phosphatidylinositol (Hirasawa and Nishizuka 1985). Agents which inhibit the PLA_2 dependent metabolism of phospholipids have also been reported to inhibit the activation of neutrophil NADPH oxidase (Maslen et al 1987).

In this study the effects of three PLA_2 inhibitors, human recombinant lipocortin 1, pBPB and mepacrine (an antimalarial drug) were investigated on the stimulated and basal oxidative metabolism of human neutrophils. Stimulus specific inhibition was observed with lipocortin and pBPB. Both basal and FMLP stimulated H_2O_2 production were inhibited by these agents. The PMA response was not significantly inhibited by either agent and H_2O_2 stimulated by aggregated IgG was only inhibited at high concentrations of pBPB. The effects of mepacrine, were not stimulus specific. Basal H_2O_2 production was very sensitive to inhibition by mepacrine whilst stimulated responses were only partially inhibited by this agent (10 fold higher concentration being required to produce the same inhibiton of stimulated H_2O_2 production as that for basal H_2O_2). This trend was also observed for the other two PLA_2 inhibitors but to a lesser degree. The lack of stimulus specific inhibition at high concentrations of mepacrine may be related to its ability to directly inhibit the hexose monophosphate shunt (HMP) (Ferrante et al 1986). HMP activity is responsible for generating a continous supply of NADPH. In accordance with this it would be expected that any reaction utilising NADPH would effectively be inhibited by mepacrine. Since the stimulation of neutrophil oxidative metabolism by any agent requires a continuous source of electrons (ie from NADPH) all would be susceptible to the effects of mepacrine. Another possibility for the lack of mepacrines stimulus specific activity

would be a direct antioxidant effect. In these studies mepacrine (at concentrations up to 1 x 10^{-4}M) had no significant effect upon superoxide production generated by a xanthine/xanthine oxidase system or on phenol red oxidation induced by 5 x 10^{-5}M reagent H_2O_2. A direct antioxidant effect could therefore be discounted. Antimalarial drugs have also been reported to have non-specific effects on membrane fluidity. Mepacrine, however, binds poorly to phospholipids and it is unlikely therefore that at the concentrations employed in this study that this effect would contribute to its inhibitory activity on NADPH oxidase (Ferrente et al 1986).

To confirm the antiphospholipase activity of these agents their effects on basal and stimulated arachidonic acid release were investigated in cells prelabelled with ^{14}C arachidonic acid. Arachidonic acid (AA) is preferentially incorporated into the 2 position of the glycerol moiety of phospholipids. Hirata et al (1979) has reported that 65% of the ^{14}C-AA taken up by neutrophils was incorporated into phosphatidylcholine and about 25% into phosphatidylethanolamine and its derivatives.

Spontaneous and PMA stimulated ^{14}C-AA release were similar to that reported by other workers (ie 7% and 0% respectively) (Dr L Steel, Boots Pharmaceuticals, personal communication). In contrast to this FMLP stimulated only very low levels of ^{14}C-AA release and aggregated IgG did not increase release over basal levels. The poor response to both these agents may be related to the lack of extracellular calcium in the medium. In the presence of extracellular calcium aggregated IgG has been reported to stimulate calcium influx by cross linking Fc receptors (Goldstein et al 1975). Furthermore, under these conditions aggregated IgG has also been

shown to stimulate significant arachidonic acid release (Sakata et al 1987). Thus, with regard to neutrophil stimulation by this agent there appears to be a dissociation between the calcium requirement for the activation of NADPH oxidase and the metabolism of phospholipids by the PLA_2 pathway. Extracellular calcium being required for the latter.

The small rise in ^{14}C-AA release following neutrophil stimulation with FMLP may be due to the mobilization of calcium from intracellular stores (Korchak et al 1984). It is of interest to note that both FMLP and aggregated IgG stimulated considerably lower levels of H_2O_2 in these studies than reported by other workers. Addition of extracellular calcium to neutrophils is known to enhance the FMLP response (Smolen et al 1981) and it would be of interest to see if a similar enhancement would also be observed with aggregated IgG. If so, it might be expected that ^{14}C-AA release stimulated by these agents would increase if this were related to the influx of calcium ions. The inhibitory effects of the PLA_2 inhibitors on the oxidative response to FMLP but not aggregated IgG support this possibility. FMLP stimulated H_2O_2 production was inhibited by these agents. Therefore the small rise in intracellular calcium and subsequent activation of PLA_2 appears to be important for the stimulation of NADPH oxidase by FMLP. Addition of extracellular Ca^{2+} to neutrophils stimulated with aggregated IgG may well reveal a PLA_2 dependent response. Certainly monocytes stimulated under these conditions exhibit an oxidative response sensitive to PLA_2 inhibition (Sakata et al 1987). However, Godfrey et al (1987) have shown that aggregated IgG stimulates superoxide production in a PLA_2 independent manner.

In the light of the ability of calcium ions to increase basal H_2O_2 production, it would be interesting to determine if changes in extracellular calcium were paralleled by increased ^{14}C AA release. Goldstein et al (1974) have demonstrated the ability of calcium ions in the absence of other stimuli to provoke the exocytosis of granule-associated lysosomal enzymes. Although it has been reported that extracellular Ca^{2+} can interact with membrane phospholipids it would seem unlikely that this per se would lead to PLA_2 and NADPH oxidase activation. However, it has been suggested that extracellular calcium provokes membrane-membrane adherence by acting as an intermembrane bridge to mediate cell production in the absence of a stimulus (Vollet et al 1972). The lack of inhibition of basal ^{14}C-AA release by PLA_2 inhibitors would indicate that arachidonic acid was being released from a PLA_2 insensitive pool, possibly from PI (Godfrey et al 1987) PMA stimulated H_2O_2 was not significantly inhibited by lipocortin or pBPB at even the higest concentrations. This was expected in view of the fact that PMA responses do not appear to require elevated intracellular calcium levels or PLA_2 activation. It has been reported that PMA stimulated H_2O_2 production occurs solely through the activation of protein kinase C (Tauber 1987).

Unfortunately supplies of lipocortin were insufficient to complete the inhibitor response curve. However it is unlikely that total inhibition of H_2O_2 production would have occurred even at concentrations which completely block PLA_2 activation since FMLP has also been reported to activate NADPH oxidase through PKC dependent mechanisms. Thus for FMLP, at least two mechanisms of NADPH oxidase activation can occur. The classical route involves the generation of diacylglycerol from PIP_2

and activation of PKC; thus mimicking the action of PMA. Alternatively, the generation of IP_3 subsequent to PLC activation may lead to the mobilization of intracellular calcium, activation of PLA_2 through calmodulin dependent enzymes and stimulation of NADPH oxidase by another mechanism, possibly by altering membrane fluidity. The extent to which each pathway is involved may well depend upon the presence or concentration of extracellular calcium. The mechanism by which aggregated IgG stimulates NADPH oxidase in the absence of extracellular calcium remains obscure but does not appear to involve PLA_2.

The final section of this chapter discusses at the role of PLA_2 in the chemotatic responses of neutrophils. Two aspects of chemotaxis were investigated, cell polarisation and orientated migration.

Polarising responses were obtained with FMLP and LTB_4, with maximum polarisation by both agents being obtained at 10^{-8}M. Cytochalasin B (CB) an agent reported to inhibit leukocyte locomotion substantially reduced the polarising responses to both stimuli. Neutrophils treated with .5 ug/ml CB became spherical and lost the ruffled appearance of their membranes. Similar effects with CB have been reported with other cells (Carter 1967, Zigmond and Hirsch 1972). The effects of the PLA_2 inhibitors were variable. At concentrations of lipocortin and pBPB employed to inhibit H_2O_2 production no inhibition of polarisation occurred, regardless of the stimulant. Mepacrine, in contrast inhibited polarising responses to both stimuli. Mepacrine inhibits phospholipid methylation as well as PLA_2 activity and this may underlie its effects on polarisation. PLA_2 activity per se does not appear to be important in the polarising

responses to LTB_4 and FMLP. The effects of PLA_2 inhibitors on orientated migration were similar to those on the polarisation response ie inhibition by mepacrine but not lipocortin or pBPB. The results with lipocortin are in direct contrast to those of Schiffmann et al (1984) who found that lipocortin inhibited chemotactic responses to FMLP. However Schiffmann did not compare the effects of lipocortin with other PLA_2 inhibitors so caution must be expressed in interpreting his results.

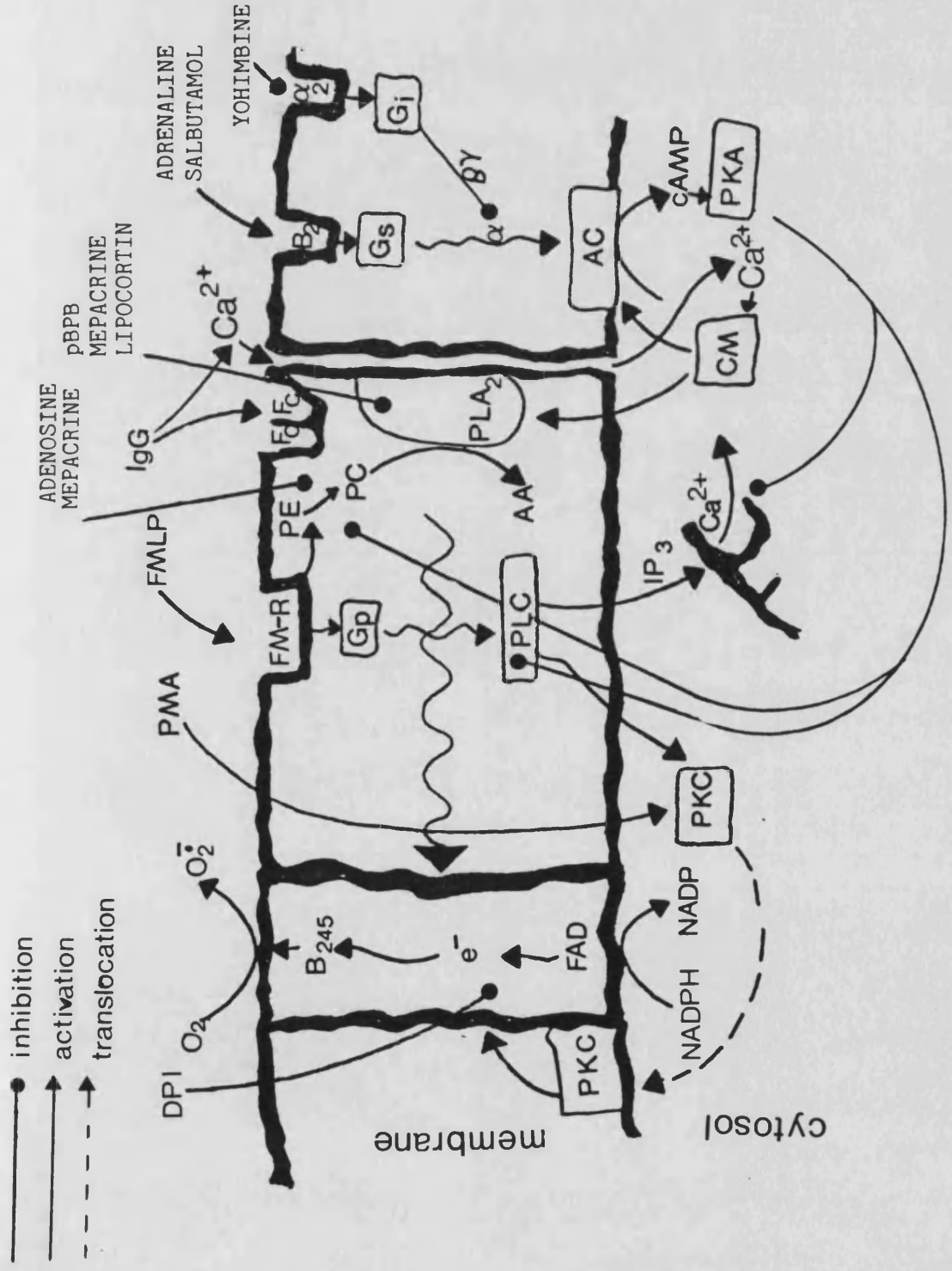
Of great interest in Schiffmann's paper was the observation that adenosine inhibited phospholipid methylation but not chemotaxis. The inhibitory effects of adenosine on H_2O_2 production noted in this study were atypical of an agent acting through the stimulation of adenylate cyclase and cAMP. Inhibition profiles by this agent however, were however remarkably similar to pBPB and lipocortin, ie indicative of PLA_2 inhibition. By inhibiting phospholipid methylation it may be argued that adenosine reduces the availability of membrane phosphatidylcholine and inhibits PLA_2 by substrate depletion. Hirata et al (1979) have shown that stimulated PLA_2 activity in neutrophils can be inhibited by raising intracellular levels of S-adenosyl homocysteine an inhibitor of phospholipid methyl transferase. This agent prevents the formation of phosphatidylcholine and so inhibits PLA_2 activity by substrate depletion.

The results presented in this thesis clearly demonstrate that distinct molecular mechanisms are involved in activating NADPH oxidase by different stimuli. Stimulation of oxidative metabolism by FMLP involves in part a PLA_2 dependent step. H_2O_2 production stimulated by IgG does not involve PLA_2 activation when neutrophils are stimulated without

extracellular calcium. In the presence of extracellular calcium, influx does occur and a calmodulin/PLA_2 dependent stimulation of NADPH oxidase may result (Sukata et al 1987). PMA stimulation which does not result in a detectable rise in intracellular calcium (Rink et al 1981) and appears to be exclusively mediated through protein kinase C (Tauber et al 1987).

Basal H_2O_2 production was sensitive to the effects of adenosine and the PLA_2 inhibitors. Both mepacrine and adenosine inhibited basal H_2O_2 indicating that phospholipid methylation may be important for H_2O_2 production in 'resting cells'. That mepacrine was considerably more potent at inhibiting basal than FMLP stimulated H_2O_2 production is not entirely unexpected. FMLP stimulation is accompanied by increased phospholipid methylation and since mepacrine inhibits this, a direct competition may well be occurring that ultimately reduces the effect of this inhibitor. The inability of PLA_2 inhibitors (other than mepacrine) to inhibit chemotaxis in FMLP treated neutrophils indicates that different molecular mechanisms of neutrophil activation might be related to the ultimate response as well as the nature of the stimulant.

Figure 44 - THE MOLECULAR MECHANISMS LEADING TO NADPH OXIDASE ACTIVATION AND THEIR MODULATION BY PHARMACOLOGICAL AGENTS



Neutrophil Derived Oxidants and Microvascular Damage in Connective Tissue Diseases.

The data presented in Chapter 6 indicates that neutrophils from patients with SS, MCTD, SLE and probably primary Raynauds are in an activated state: ie they generate significantly more basal H_2O_2 than healthy subjects. Furthermore, inter-group differences in neutrophil H_2O_2 production were paralleled by their levels of serum FVIII Rag. Therefore for each disease group as a whole the degree of microvascular damage appeared to be related to neutrophil activity. Whether the state of activation of neutrophils in the disease groups is the result of prior stimulation in vivo is unknown. In this respect potential stimulants could be immune complexes (IC) (Henson and Oades 1975). Although uncommon in SS (less than 20% occurrence - PJ Maddison - personal communication) IC's figure prominently in the vascular pathology of SLE (Lambert and Casalli 1978). Via et al (1984) have demonstrated the direct stimulation of luminol-dependent chemiluminescence (an indicator of H_2O_2 production) in normal human neutrophils by sera from patients with SLE. The stimulatory capacity of the serum correlated well with serological indices of disease activity and in particular the presence of Clq binding immune complexes (Hurd et al 1980). Anti-DNA immune complexes have been implicated in the pathogenesis of vasculitis and nephritis in SLE (Lloyd and Schur 1981). In the present study SLE patients were either clinically inactive or only mildly active. Therefore both H_2O_2 production and FVIII Rag levels appear to be unrelated to clinical disease activity. The lack of correlation between FVIII Rag and disease activity in SLE has been reported by other workers (Belch et al 1987). Seventy percent of SLE patients, however, were seropositive for anti-DNA antibodies as detected by radioimmunoassay

and mean basal H_2O_2 production by neutrophils from these patients was higher than from the seronegative group but this was not statistically significant. Factor VIII Rag levels were also high in patients with anti-DNA antibodies (149 ± 40%, n=8) but there was insufficient data from the seronegative group for comparison. The onset of lupus nephritis is associated with marked changes in circulating levels of DNA (antigen) and anti DNA antibodies. When accompanied by the activation of complement this may well be indicative of immune complex formation and deposition (McClusky et al 1978). Immune complexes are powerful stimulants for neutrophils (Weiss and Ward 1982) and the leukopaenia and increased expression of C3 receptors on neutrophils from patients with active SLE certainly point to neutrophil activation (Abramson et al 1986). These studies have indicated functional neutrophil activation in SLE as measured by enhanced basal H_2O_2 production. It is therefore interesting to speculate that deposition of anti DNA-immune complexes in the microcirculation and local stimulation of neutrophils to generate H_2O_2 cause vascular injury and increased levels of FVIII Rag. The increased levels of circulating immune complexes or complement derived peptides on the other hand as reported by Abramson (1987) may result in the intravascular activation of neutrophils and the observed increase in basal H_2O_2 production in vitro (Abramson et al 1987).

Elevated basal and stimulated H_2O_2 production were observed in the MCTD group. Patients with MCTD present symptoms common to both SS and SLE and are seropositive for the U_1RNP autoantibody (Sharp et al 1972). Negoro et al (1987) have concluded that the presence of U_1RNP immune complexes in sera from MCTD patients is associated with clinical disease

activity. Some patients with SLE (particularly those with Raynauds phenomenon) also have high titres of U_1RNP autoantibodies (Kurata et al 1978). No relationship, however, has been found between levels of U_1RNP-IC and disease activity in SLE (Negoro et al 1987). In the SLE group no significant differences were found with regards to levels of FVIII Rag between U_1RNP seropositive and seronegative patients (146 and 150% respectively). Both groups had significantly higher levels of FVIII Rag than the healthy control group. Three out of the five U_1RNP positive patients were seronegative for anti DNA antibodies but had high levels of Factor VIII Rag. Therefore, it would be of interest to determine whether this was a trend common to SLE anti U_1RNP+/anti DNA- patients. Certainly, within the MCTD group U_1RNP antibodies appear very much to be associated with elevated FVIII Rag levels and therefore probable microvascular damage. Whether U_1RNP antibodies or immune complexes are contributing to the elevated factor VIII Rag levels in SLE would be of interest.

A major problem with the proposal that enhanced basal neutrophil activity is the result of prior stimulation in vivo is that one would expect a reduced response to subsequent cell stimulation in vitro. H_2O_2 production stimulated by aggregated IgG in the SLE group did not differ significantly from control subjects whilst those in the SS and MCTD groups were increased. Furthermore, in the latter groups, the patients with enhanced basal activity generated higher levels of H_2O_2 following stimulation with aggregated IgG. This would suggest that rather than being stimulated in vivo the cells were in fact being primed. In this case elevated basal H_2O_2 production may be an artefact of the purification procedure which

may just highlight the subsequent sensitivity of the cells to stimulation. Because no significant differences in PMA stimulated H_2O_2 production were observed between SS groups and control it seems unlikely that the overall capacity of the cells to produce hydrogen peroxide in each group was different. Before similar observations have been reported by other workers (Maslen 1986, Gzirjak et al 1987). In contrast to this result Kovacs et al (1986) found elevated PMA responses in the SS neutrophils. The concentration of PMA employed by his group for stimulation were much higher than used in this study. Since the concentrations used here were based on the EDqo (concentration of stimulus to produce a 90% maximal response) values of control patients it may well be that SS neutrophils do have the capacity to generate more H_2O_2, in response to PMA albeit at a higher concentration. Thus, prior stimulation in vivo need not necessarily result in a reduced response to stimulants in vitro. The possibility of in vivo activation is further supported by the finding that a serum factor from SS patients is able to elevate basal and stimulated oxidative metabolism in normal healthy neutrophils (Kovacs et al 1986). The nature of this serum factor remains a mystery.

The neutrophil H_2O_2 responses to FMLP stimulation in the SS group were very interesting. Initially no significant differences were found between control and SS neutrophils as a whole. However, when the SS group was subdivided by the extent of skin fibrosis, ie that limited to sclerodactyly, and those with more diffuse skin involvement, neutrophils from patients with limited fibrosis generated significantly more H_2O_2 than control cells. Furthermore, the same group of patients generated more basal H_2O_2 than patients with diffuse fibrosis. Analysis of the IgG stimulated responses

revealed a similar pattern of neutrophil activity. These observations are in direct contrast to those reported by Gzirjak et al (1987) who found that increased neutrophil H_2O_2 production was most pronounced in patients with more extensive skin involvement.

The enhanced neutrophil activity in the sclerodactyly group is surprising if one believes that extensive fibrosis represents a more advanced stage of the disease. Two explanations may account for these findings:- first, patients with diffuse fibrosis might have more active cells but they are marginated to the endothelium and therefore not collected in the blood samples or secondly, the disease may have progressed beyond neutrophil involvement. Despite the apparent variation in neutrophil activity no differences in the level of microvascular damage were observed (with regard to mean F VIII Rag levels) between the sclerodactyly and diffuse fibrosis group. However, elevated levels occurred at a higher incidence in the sclerodactyly group (77%) than in the group with diffuse fibrosis (53%). Mononuclear cells are a common component of chronic inflammatory and fibrotic reactions may also contribute indirectly to elevated factor VIII Rag levels in the diffuse fibrosis group. In this case a study on the activation state of monocytes in SS is warranted.

Serum levels of FVIII Rag were elevated in the SS group (Lee et al 1985, Belch et al 1987). Lee (1985), has reported that the severity of vascular abnormalities noted on capillary microscopy correlated well with the levels of FVIII Rag. He found that patients with longer disease duration and diffuse SS had higher levels of circulating FVIII Rag. In contrast, no differences in FVIII Rag were noted in patients with sclerodactyly and

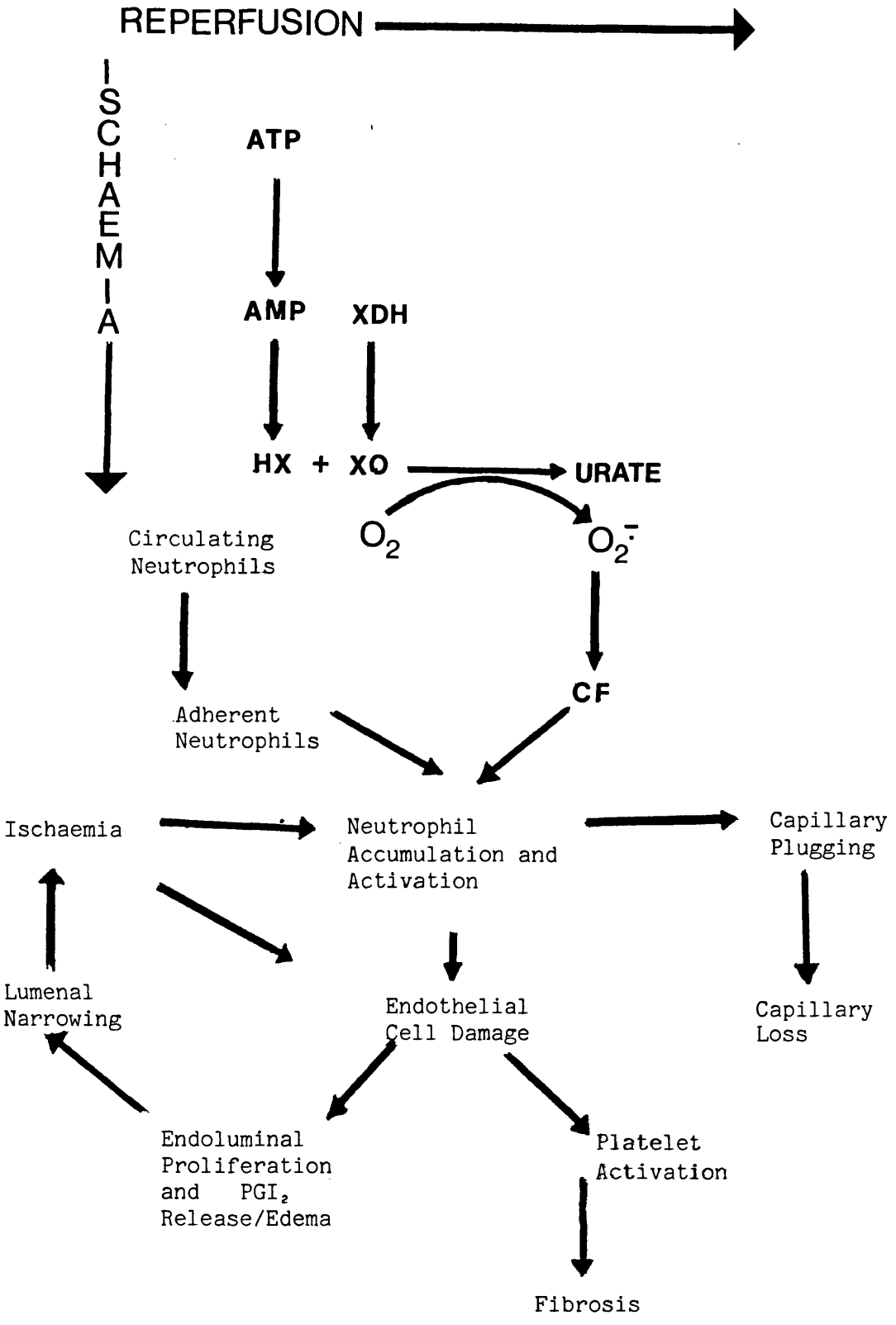
more diffuse SS in this study. This would indicate that although vascular damage may contribute to the formation of the fibrotic lesion, its extra-vascular development is controlled by other factors.

An important question to ask in the light of these studies is whether neutrophil derived H_2O_2 is responsible for the enhanced factor VIII levels in patients. Several reports have confirmed the participation of H_2O_2 or products derived from it in endothelial cell injury (Sarks et al 1978, Weiss et al 1981, Fox 1984, Ager and Gordon 1984). Ager and Gordon (1984) have reported that reagent H_2O_2 at concentrations lower than 0.1 uM prevent endothelial cells responding to vasoactive agents whilst gross structural damage required concentrations above 300 uM. The basal and stimulated H_2O_2 levels measured in patient groups in this thesis are obviously insufficient to cause direct cytotoxicity to endothelial cells. However, on a background of increased myeloperoxidase release, as reported here, the cytotoxic potential of H_2O_2 might be considerably enhanced. A report by Johnson et al (1987) have shown that in the presence of MPO subcytotoxic concentrations of H_2O_2 become cytotoxic. Cellular injury by H_2O_2 in the presence of MPO results from the formation of hypochlorous acid which can mediate lethal cellular oxidation and halogenation reactions. When SS patient F VIII Rag levels were compared with basal neutrophil H_2O_2 production no direct relationship was observed. This is not surprising since blood samples for sera and neutrophil studies were not collected on the same day. Further studies are therefore warrented to find the full relationship between neutrophil activity and FVIII Rag levels.

The prevalence of Raynauds phenomenon in SS is striking. In a small number of Raynauds patients without underlying connective tissue disease (Primary Raynauds) elevated basal H_2O_2 production was measured ($p<0.01$). Furthermore FVIII levels in a large group of Raynaud's subjects (n=65) were raised significantly above control. Longitudinal studies have shown that the development of SS from idiopathic Raynauds in accompanied by a significant rise in circulating levels of FVIII Rag (Belch et al 1987). It would be extremely interesting to run a similar longitudinal study measuring both factor VIII Rag levels and neutrophil activity in patients progressing from idiopathic Raynauds to systemic sclerosis. A parallel increase in both parameters may well shed light on the pathogenesis of the disease.

With this in mind and the data accumulated by this study and others the following model for the pathogenesis of SS is proposed:

Figure 45 REPERFUSION INJURY AS A MODEL FOR THE PATHOGENESIS OF SYSTEMIC SCLEROSIS



The Pathogenesis of Systemic Sclerosis - A Reperfusion Injury?

Early SS is invariably associated with Raynauds phenomenon and inflammation of the distal extremities (Ruggierei and LeRoy 1986). Perivascular fibrosis is apparent in these areas and appears to precede the involvement of the skin (Kahaleh and Jimenez et al 1979). Thus it is interesting to speculate that the early SS vascular lesion may result from a Raynauds induced reperfusion type injury. Certainly the vasospasm that accompanies a severe Raynauds attack can result in an area of localised ischaemia (Prof P Dieppe, University of Bristol, personal communication). In the classical reperfusion injury model (Engler 1987) neutrophils trapped within a region of ischaemia adhere to vascular endothelium and become activated. Whether hypoxia itself is a stimulus for neutrophil activation or whether endothelial products are responsible is unknown. Oxidants generated by activated neutrophils and from the ischaemic tissue following reperfusion contribute to the further accumulation of neutrophils by promoting the formation of serum chemotactic factors (Petrone et al 1980). Subsequent activation of these cells lead to the release of lysosomal enzymes and oxidant which may cause significant cell injury (McCord 1985). The response of the microvasculature to such an insult tends to exacerbate the situation. When exposed to oxidants, endothelial cells increase their production of prostacyclin (Ager and Gordon 1984, Harlan and Callahan 1984). Furthermore damaged endothelium is replaced by proliferating cells derived from smooth muscle which exhibit enhanced basal prostacyclin production. Elevated prostacyclin levels result in the extravasation of vascular fluid and an increased extravascular pressure. On the background on endothelial proliferation the rise in extravascular pressure can lead to partial or

complete occlusion of the vessel (ie worsen or prolong the ischaemia). Elevated prostacyclin production by endothelial cells in SS is apparent by the increased levels of circulating PG $F_1\alpha$ (prostacyclin's inactive metabolite) in sera from these patients (Horrobin et al 1983). At a clinical level two observations may be indicative of enhanced PGI_2 production:- first, the presence of an early oedematous phase of the disease and secondly the poor response to treatment of the Raynauds phenomenon in SS patients and the frequent occurrence of digital ulcers and infarcts (secondary to lumenal obstruction) (Ruggieri and LeRoy 1986). Elevated levels of PGI_2 or its metabolites may also be responsible for the reduced sensitivity of various cells in SS to a prostacyclin analogue in vitro (Kirby et al 1980, Belch et al 1985). In these studies a reduced response to the inhibitory effects of salbutamol on IgG stimulated H_2O_2 production was observed in SS neutrophils. This may represent a pharmacological down-regulation of the B receptor-linked cAMP generating system in these patients. Similar down-regulation phenomena have been reported in other diseases (Sulser 1981). Agents which elevate cAMP levels were found to inhibit basal H_2O_2 production in this study. It is therefore possible that low levels (basal levels) of cAMP may regulate the spontaneous activity of NADPH oxidase. Down-regulation of the cAMP generating system in the way described above may then lead to a defective control of NADPH oxidase and the increased basal and stimulated H_2O_2 production seen in the SS patients.

Damage to small arteries and arterioles are a common feature of the vascular pathology of SS (Kahaleh et al 1979). This is not entirely unexpected since these vessels normally have a rich oxygen supply and

are therefore much more susceptible to ischaemic injury than the venous circulation (Ryan 1980). In myocardial reperfusion injury accumulation of neutrophils during ischaemia may lead to capillary plugging and a no-reflow phenomenon (Schmid-Schonbien 1987). Capillary shut down follows and then degeneration. Certainly the loss of capillaries is a common pathological feature of SS (Kahaleh et al 1979).

An important question to ask is how endothelial cell damage can lead to skin fibrosis. From a histological viewpoint, perivascular fibrosis precedes skin involvement in early SS. Neutrophil mediated oxidative damage to endothelial cells can lead to the exposure of subendothelium, a powerful stimulus for platelet adhesion and activation. On a background of increased endothelial prostacyclin production one would expect platelet activation to be reduced. However, platelets from SS patients are relatively refractory to prostacyclin and appear to be hyper-adherent in vitro (Kahaleh et al 1985). Activated platelets release factors capable of stimulating fibroblast mitogenesis, including PDGF (Blanchardet et al 1985). This, together with reports of reduced collagenase activity in SS may result is excessive deposition of collagen in the skin and the characteristic cutaneous manifestations of the disease. Thus, localised tissue fibrosis, eg sclerodactyly, may result from repeated ischaemic insults or a maintained ischaemia in the region of the hands whilst the intravascular activation of neutrophils may lead to their margination in other regions of the circulation and subsequently to the damage of visceral organs (eg lung and kidney).

The observation that FVIII Rag levels were elevated in patients with silica induced SS is interesting and raises the possibility that environmental factors may predispose to development of the disease. Of 18 patients in this group 9 (50%) were seropositive for autoantibodies to Scl 70. This autoantibody is generally associated with diffuse SS and is rarely found in patients with sclerodactyly (Riboldi et al 1985). The hypothesis proposed above readily explains the development of sclerodactyly from a Raynauds reperfusion injury. However it may be that environmental factors in addition to the presence of Raynauds phenomenon lead to the development of the more diffuse forms of SS.

References

Abramson S, Buyon J and Shaddick N (1986).
Surface expression of CR3 on neutrophils as a marker of disease activity is systemic lupus erythematosus.
Clin Res 34 614A (abst).

Abramson S, Belmont HM, Hopkins P, Buyon J, Winchester R and Weissmann G (1987).
Complement activation and vascular injury in systemic lupus erythematosus.
J Rheumatol 14 43-46.

Alobaidi T, Naccache PH and Shaafi RI (1981).
Calmodulin antagonists modulate neutrophil degranulation, aggregation and stimulated oxygen consumption.
Biochem Biophys Acta 675 316-321

Anderson CL, Guyre PM, Whitin JC, Ryan DH, Looney RJ and Fanger MW (1986).
Mononuclear antibodies to Fc receptors for IgG on human mononuclear phagocytes. Antibody characterisation and induction of superoxide production in a monocyte cell line.
J Biol Chem 261 12856-12864.

Anderson R and Jones PT (1982).
Increased leuko-attractant binding and reversible imhibition of neutrophil motility mediated by the peroxidase/H_2O_2/halide system: effects of ascorbate cysteine, dithiothreitol, levamisole and thiamine.
Clin Exp Immunol 47 487-496.

Anderson DC, Schmalsteig FC, Finegold NF, Hughes BJ, Rothlein R and Miller LJ (1985).
The severe and moderate phenotypes of heritable MAC-1 and LFA-21 defficiency: their quantitative dysfunction and relation to leukocyte dysfunction and clinical features.
J Infect Dis 152 668-673

Anderson DC, Miller LJ, Schmalsteig FC, Rothlein R and Springer TA (1986).
Contribution of the MAC-1 glycoprotein fairly to adherence dependent granulocyte function: Structure function assessment employing subunit specific monoclonal antibodies.
J Immunol 137 15-27.

ARA (1980).
Preliminary criteria for the classfiication of systemic sclerosis (scleroderma).
Arth Rheum 23 581-590.

Aust SF, Morehouse LA and Thomas CE (1985).
Role of metals in oxygen radical reactions.
J Free Radical Biol Med 1 3-25.

Babior GL, Rosin RE, McMurrich BJ, Peters WA, and Babior BM (1981).
Arrangment of the respiratory burst oxidase in the plasma membrane of the neutrophil.
J Clin Invest 67 1724-1728.

Babior BM (1978).
Oxygen dependent killing by phagocytes.
New Eng J Med 298 659-668.

Babior BM and Kipnes RS (1977).
Superoxide-forming enzyme from human neutrophils: Evidence for a flavin requirement.
Blood 50 517-524.

Babior BM, Curnutte JT and McMurrich BJ (1976).
The particulate superoxide forming system from human neutrophils: Properties of the system and further evidence supporting its participation in the respiratory burst.
J Clin Invest 58 989-996.

Babior BM, Crowley CA, Rosin RE, Buchanan AR and Crunbrone MA (1981).
GP180 deficiency: An abnormality in specific granules as the cause of a defect in adherence.
Blood 588 669 (abst).

Bagby GC, Rigas VD, Bennet RM, Vanderbark AA, Garewell HS, Wilkinson B and Davis J (1981).
Interaction of lactoferrin, monocytes and T-lymphocyte subsets in the regulation of steady state granulopoiesis in vitro.
J Clin Invest 68 65-63.

Baggiolini M and Dewald B (1984).
Endocytosis by neutrophils.

In current topics in Immunobiology. Regulation of leukocyte function. ed R Snyderman, Plenum Press, New York.

Bainton DF and Farquhar MG (1968).
Differences in enzyme content of azurophyl and specific granules of polymorphonuclear leukocytes. II cytochemistry and electron microscopy of bone marow cells.
J Cell Biol 39 299-317.

Baldridge CN and Gerard RW (1933).
The extra respiration of phagocytosis.
Am J Physiol 103 235-245.

Baumgartner HR (1985).
The subendothelial surface and thrombosis.
In Proceedings of the 4th Congress of the International Society on Thrombosis and Haemostasis, Vienna 42.

Becker EL, Kermode JC, Naccache PH, Yassin R, Munoz JJ, Marsh ML, Huang CK and Shaafi RI (1986).
Pertussis toxin as a probe of neutrophil activation.
Fed Proc 45 2152-2155.

Becker EL, Showell HJ, Naccache PH and Shaafi RI (1981).
The role and inter-relationships of Ca^{2+} and arachidonic acid metabolism in stimulated neutrophil functions.

Belch JJF, Zomma AA, Richards IM, McLaughlin K, Forbes CD and Sturrock RD (1987).
Vascular damage and factor VIII related antigen in the rheumatic diseases.
Rheumatol Int 7 107-111.

Belch JJF, O'Dowd A, Forbes CH and Sturrock RD (1985).
Platelet sensitivity to a prostacyclin analogue in systemic sclerosis.
Br J Rheumatol 24 346-350.

Bellavite P, Papini E, Zeni L, Della Bianca V and Rossi F (1985).
Studies on the nature and activation of $O_2^{-\cdot}$ forming NADPH oxidase of leukocytes: identification of a phosphorylated component of the active enzyme.
Free Rad 5 Comm 1 11-29.

Berridge MJ (1981).
Receptors and calcium signalling.
In Towards Understanding Receptors pp 122-131.
ed Lamble JW, Elsvier, NOAL, Holland.

Bielski BHJ, Arudi RL and Sutherland MW (1983).
A study of the reactivity of $HO_2/O_2^{-\cdot}$ with unsaturated fatty acids.
J Biol Chem 258 4759-4761.

Biemond P, Herk G, Van Ejik A, Swaak JG and Koster JF (1984).
Iron mobilization from ferritin by $O_2^{-\cdot}$ derived from stimulated polymorphonuclear leukocytes.
J Clin Invest 73 1576-1579.

Biemond P, Swaak AJG, Beinorff CM and Kosher (1986).
Superoxide dependent and independent mechanisms of iron mobilisation from ferritin by xanthine oxidase.
Biochem J 239 169-173.

Blackwell GJ, Carnuccio R, Dirosa M, Flower RJ, Parente L and Persico P (1980).
Macrocortin, a polypeptide causing the antiphospholipase effect of glucocorticoids.
Nature 287 147-149.

Blake DR, Winyard P, Scott DGI, Brailsford S, Blann A and Lunec J (1984).
Endothelial cell cytotoxicity in inflammatory vascular diseases - the possible role of oxidised lipo proteins.
Ann Rheum Dis 44 176-182.

Blanchard JM, Piechaczyk M, Dani C.
C myc gene is transcribed at hight rate in Go arrested fibroblasts and is post transcriptionally regulated in response to growth factors.
Nature (Lond) 317 443-6.

Bormann BJ, Huang CK, Mackin WM and Becker EL (1984).
Receptor mediated activation of phospholipase A_2 in rabbit neutrophil plasma membrane.
Proc Nat Acac Sci 81 767-770.

Boto K, Tamura H, Nagayama M (1970). The mechanism of oxygenation of ferrous ion in neutral solutions.
Inorg Chem 9 963-64.

Boyden S (1953).
Chemotactic effect of mixtures of antibody and antigen.
J Exp Med 115 453.

Brady AH (1975).
Collagenase in scleroderma.
J Clin Invest 56 1175-1180.

Bradford PG and Rubin RP (1985).
Pertussis toxin inhibits chemotactic factor induced phospholipase C stimulation and lysosomal enzyme secretion in rabbit neutrophils.
FEBS 183 317-320.

Burger RM, Peisch J and Horivitz SB (1981).
Mechanisms of bleomycin action in vitro studies.
Life Sci 28 715-727.

Butcher EC, Hewinsohn D, Duiyvestyin A, Bargalzer R, Nill N and Jolanen S (1986).
The interaction between endothelial cells and leukocytes. J Cell Biochem 30 121-131.

Carp H and Janoff A (1979).

In vitro suppression of serum elastase inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. J Clin Invest 63 793-797.

Carp H and Janoff A (1980).
Potential mediators of inflammation. Phagocyte derived oxidants suppress the elastase inhibitory capacity of alpha 1 proteinase inhibitor in vitro. J Clin Invest 66 987-995.

Castor CW, Ritchie JC, Williams CH (1979).
Connective tissue activation XIV composition and actions of a human platelet autocoid mediator.
Arthritis Rheum 22 260-272.

Chance B, Sies H and Borens A (1979). Hydroperoxide metabolism in mammalian organs. Physiol Rev 59 527-605.

Clark RA (1982).
Chemotactic factors trigger their own oxidative inactivation by human neutrophils.
J Immunol 129 2725-2728.

Cronstein BN, Kramer SB, Weissmann G and Hirschlom R (1983).
Adenosine: A physiological modulator of superoxide anion generation in human neutrophils.
J Exp Med 158 1160-1177.

Cohen HJ and Chovaniec ME (1978).
Superoxide generation by digitonin stimulated guinea pig granulocytes.
J Clin Invest 61 1081-1086.

Cohen P (1985).
The role of protein phosphorylation in the homonal control of enzyme activity.
Eur J Biochem 151 439-448.

Cohen S, Johnson AR and Hurd E (1983).
Cytotoxicity of sera from patients with scleroderma.
Arthrtis Rheum 26 170-178.

Cross AR and Jones OTG (1986).
The effect of diphenylene iodonium on the superoxide generating system of neutrophils.
Biochem J 237 111-116.

Crutti P (1985).
Pro-oxidants state and tumour promotion.
Science 227 375-381.

Cunningham CC, DeChalelet LR, Spach PI, Parce JW, Thomas MJ, Lees CJ and Shirley PS (1982).
Identification and quantitation of electron transport components in human polymorphonuclear neutrophils.
Biochim Biophys Acta 682 430-435.

Curnutte JT, Babior BM (1974).
Biological defense mechanisms. The effects of bacteria and serum on superoxide production by granulocytes. J Clin Invest 53 1662-1672.

Czirjak L, Danko K, Sipka S, Zeter M and Szecjedi G (1987).
Polymorphonuclear neutrophil function in systemic sclerosis.
Ann Rheum Dis 46 302-306.

Dahlen SE, Bjork J, Hedquist, Arforske Hammerstrom S, Lindgren JA and Samuelsson B (1981).
Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules. In vivo effects with relevance to the acute inflammatory response.
Proc Natl Acad Sci USA 78 3887-3891.

D'angelo WA, Fries JF and Masi A (1969).
Pathological observations in systemic sclerosis (scleroderma).
Am J Med 46 428-440.

Davidson FF, Dennis EA, Powell M and Glenney JR (1987).
Inhibition of phospholipase A_2 by lipocortins and calpactins. The effect of binding to substrate.

Davies KJA and Goldberg A: (1987).

Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes.
J Biol Chem 262 8220-8226.

Davies JM and Williams KI (1984).
Endothelial dependent relaxant effects of vasoactive intestinal peptide and arachidonic acid in rat aortic strips.
Prostaglandins 27 195-

Davies KJA (1987).
Protein damage and degradation by oxygen radicals.
J Biol Chem 262 9895-9901.

Davies KJA, Delsignore ME and Lin SW (1987).
Protein damage and degradation by oxygen radicals: Modification of amino acids.
J Biol Chem 262 9902-9907.

De Chatalet LR (1978).
Initiation of the respiratory burst in human polymorphonuclear neutrophils: A critical review.
J Reticulo End Soc 24 73-89.

De Chatelet LR, Shirley PS and Johnson RB (1976).
Effect of phorbol mynstate acetate on the oxidative metabolism of human polymorphonuclear leukocytes.
Blood 47 545-554.

Denko CW (1979).
The protective role of ceruloplasmin in inflammation.
Agents Actions 9 333-336.

Dewald B, Baggiolini M, Curnutte JT and Babior BM (1979).
Subceilular localization of the superoxide forming enzyme in human neutrophils.
J Clin Invest 63 21-29.

Dickey BF, Pyun HY, Williamson KC and Novano J (1987).
Identification and purification of a novel G protein from neutrophils.
FEBS Letts 219 289-292.

Dixit R, Mukhtar H and Bickers DR (1982).
Evidence that lipid peroxidation in microbial membranes is associated with the generation of hydrogen peroxide and singlet oxygen.
Biochem Biophys Res Comm 105 546-552.

Dixit R, Mukhtar H and Bickers DR (1982).
Evidence that lipid peroxidation in micromal membranes of epidermis is associated with generation of hydrogen peroxide and singlet oxygen.
Biochem, Biophys Res Comm 105 546-552.

Dohlman HG, Caron MG and Lefkowitz RJ (1987).
A family of receptors coupled to guanine neucleotide regulatory proteins.
Biochemistry 26 2664-2668.

Drath DB and Karnovsky ML (1974).
Bactericidal activity of metal mediated peroxide-associated systems.
Infect Immunol 10 1077-1083.

Duque RE, Fantone JC, Kramer C, Marasco WA and Sen HP (1986).
Inhibition of neutrophil activation by p bromophenacyl bromide and its effects on phospholipase A_2.
Br J Pharmac 88 463-472.

Engelberger W, Bitter-Suermann D and Hadding U (1984).
Involvement of phospholipid metabolism in the events of the oxidative burst of granulocytes.
Agents Actions 15 31-33.

Engler R (1987).
Consequences of activation and adenosine mediated inhibition of granulocytes during mycardial ischaemia.
Federation Proc 46 2407-2412.

English D and Cabig T (1986).
Differentiation in the cell processes involved in induction a maintenance of stimulated neutrophil adherence.

Blood 67 1314-1322.

Estensen RD, White JG and Holmes B (1974).
Specific degranulation of human polymorphonuclear leukocytes.
Nature (Lond) 248 347-348.

Fantone JC and Ward PA (1985).
Antioxidant Mechanisms in Oxygen-derived Radicals and their Metabolites:
Relationship to tissue injury.
pp 15-17 Upjohn Kalamazoo.

Fantone JC and Kinnes DA (1983).
Prostaglandin E_1 and prostaglandin I_2 modulation of superoxide production
by human neutrophils.
Biochem Biophys Res Comm 113 506-512.

Fee JA and Valentine JS (1977).
Chemical and physical properties of superoxide.
In superoxide and superoxide dismutases ed Michelson M, McCord JM, and
Fridovich I.
pp 19-60 Academic Press, New York.

Ferrante A, Rowen-Kelly B, Seow WY and Thong YH.
Depression of human polymorphonuclear leukocyte function by anti malarial
drugs.
Immunol 58 125-130.

Ferreira SH and Vane JR (1976).
Prostaglandins: their disappearance from and their release into the circulation.
Nature (Lond) 216 868-871.

Finch WR, Rodnan GP, Buckingham RB, Prince BK and Winkelstein A (1980).
Bleomycin induced scleroderma.
J Rheumatol 7 651-659.

Fitzharris P, Cromwell O, Moqbel R, Hartnell A, Walsh GM, Harvey C and Kay AB (1987).
Leukotriene B_4 generation by human neutrophils following IgG dependent stimulation.
Immunology 61 449-55.

Flohe L, Martin W, Laschen G and Gunzer NA (1984).
Is leukotriene B_4 induced chemotaxis mediated by superoxide?
Life Chem Rep Suppl 2 318.

Flohe L, Giertz H and Beckmann (1985).
Free radical scavengers as anti-inflammatory drugs in The pharmacology of inflammation.
eds Bontalh, Bray MA and Parhham MJ, pp 255-281, Elserier, Oxford.

Florey HW and Grant LH (1961). Leukocyte migration from small blood vessels stimulated with UV light: an electron microscopic study. J Path Bact 82 13-20.

Ford-Hutchinson AW, Pawlowski NA, Murray HW, Andreach M, Zrike J and Cohn Z (1982).
Regulation of arachidonic acid metabolism by macrophage activation.
J Exp Med 155 1148-1153.

Freeman BA and Crapo JD (1982).
Biology of disease: Free radicals and tissue injury.
Lab Invest 47 412-426.

Frey T, Janes M, Englehardt W, Affign EG, Geerds C and Mooker B (1986).
Fc gamma receptor mediated changes in the plasma membrane.
Eur J Biochem 158 85-89.

Fridovich I (1978).
The biology of oxygen radicals.
Science 201 875-880.

Fridovich I (1983).
Superoxide radical: An endogenous toxicant.
Ann Rev Pharmacol Toxicol 23 239-257.

Furchgott RF (1981).
The requirement for endothelial cells in the relaxation of arteries by acetylcholine and some other vasodilators.
TIPS 2 173-175

Gabig TG, Schervish EW and Santinga JT (1982).

Functional relationship of cytochrome b to the superoxide generating oxidase of human neutrophils.
J Biol Chem 257 4114-4119.

Gale RJ, Nikoloutsopoulos A, Bradley J and Roberts-Thomas PJ (1985).
Immune complex activation of neutrophis and enhancement of the activation by rheumatoid factor and complement.
J Rheumatol 12 21-26.

Gallant SP and Alfred SJ (1980).
Demonstration of $beta_2$ adrenoceptor and effects of high coupling effectors in human neutrophil sonicates.
J Lab Clin Med 96 15-

Gallin J, Durocher JR and Kaplan AP (1975).
Interactions of leukocyte chemotactic factors with the cell surface 1. Chemotactic factor induced changes in human granulocyte surface charge.
J Clin Invest 55 967-

Gancedo JM, Mazon MJ and Eraso P (1985).
Biological roles of cAMP - Similarities and differences between organisms.
TIBS 210-212.

Gerrad C, McPhail LC, Marfat A, Stimer-Gerad P, Bass DA and McCall CE (1986).
Role of protein kinases in stimulation of human polymorphonuclear leukocytes oxidative metabolism by various agonists.
J Clin Invest 77 61-65.

George P (1965).
The fitness of oxygen.
In oxidases and related redox systems.
eds King TE, Mason HS, Morrison M. pp 3-36 John Willey and Sons, New York.

Gilman AG (1984).
G proteins and dual control of adenylate cyclase.
Cell 36 577-579.

Godfrey RW, Marisi RN, Clark MA and Hoffstein ST (1987).
Stimulus specific induction of phospholipid and arachidonic acid metabolism in human neutrophils.
J Cell Biol 104 925-932.

Goldman DW and Goetzl EJ (1984).
Heterogenecity of human polymorphonuclear leuukocyte receptors for leukotreine B_4.
J Exp Med 159 1027-1040.

Goldman DW and Goetzl EJ (1982).
Specific binding of leukotriene B_4 to a receptor on human polymorphonuclear leukocytes.
J Immunol 129 1600-130.

Goldstein IM, Kaplan HB, Edelson HS amd Weissmann G (1978). Cerulloplasmin: a scavenger of superoxide anion radicals. Biol Chem 254 4040-4045.

Goldstein IM, Roos D, Kaplan HB and Weissmann G (1975). Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. J Clin Invest 56 1155-1163.

Goldstein IM, Hom JK, Kaplan HB and Weissmann G (1974). Calcium induced lysosomal enzyme secretion from human poly morphonuclear leukocytes. Biochem Biophys Res Comm 60 807-812.

Goldstein IM, Cerqueira M, Lind S and Kaplan TB (1977). Evidence that the superoxide generating system of human leukocytes is associated with the cell surface. J Clin Invest 59 249-254.

Greengard P (1978). Phosphorylated proteins as physiological effectors. Science 199 146-151.

Grinstein S, Furuga W and Gragoe J (1986). Volume changes in activated neutrophils. The role of Na^+/H^+ exchange. J Cell Physiol 128 33-40.

Grinsham MB, Jefferson MM, Melton DF and Thomas EL (1984).

Chlorination of endogenous-amines by isolated neutrophils. Ammonia dependent bactericidal, cytotoxic and cytolytic activities of the chloramines.
J Biol Chem 259 10404-10413.

Gutteridge JMC, Segal AW, Patterson K, Halliwell B (1981).
Inhibition of lipid peroxidation by the iron binding protein lactoferrin.
Biochem J 199 259-261.

Gutteridge JMC (1983).
Anti oxidant properities of erucloplasmin towards iron and copper dependent oxygen radical formation.
FEBS 157 37-40.

Halliwell B and Gutteridge JMC (1984).
Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy.
Lancet 1396-1397.

Halliwell B and Gutteridge JMC (1986).
Iron and free radical reactions: two aspects of anti-oxidant protection.
TIBS 372-375.

Harrison JE and Schultz (1976).
Studies on the chlorinating activity of myeloperoxidase.
J Biol Chem 251 1321-1374.

Harlan JM and Callahan KS (1984).
Role of hydrogen peroxide in the neutrophil mediated release of prostacyclin from cultured endothelial cells.
J Clin Invest 74 442-448.

Harslam RJ and Lynham JA (1977).
Relationship between phosphorylation of blood platelet proteins and secretion of platelet granule constituents. Effects of different aggregating agents.
Biochem Biophys Res Commun 77 714-722.

Haston WS and Shields JM (1985).
Neutrophil leukocyte chemotaxis: a simplified assay for measuring polarising responses to chemotactic factors.
J Immunol Methods 81 229-237.

Hayakawa T, Suzuki K, Suzuki S, Andrews PC and Babior BM (1986).
A possible role for protein phosphorylation in the activation of the respiratory burst in human neutrophils. Evidence from studies with cells from patients with chronic granulomatous disease.
J Biochem 261 9109-9115.

Hatch CE, Gardner DE and Menzel DB (1978).
Chemiluminesence of phagocytic cells caused by N formylmethionyl peptides.
J Exp Med 147 182-195.

Helfman DM, Appelbaum BD, Volger NR and Kuo JF (1983).

Phospholipid sensitive Ca^{2+} dependent protein kinase and its substrates in human neutrophils.
Biochem Biophys Res Comm 111 847-

Henderson WR and Klebanoff SJ (1983).
Leukotriene production and inactivation by normal and myeloperoxidase defficent neutrophils.
J biol Chem 158 13522-13527.

Henson PM (1971).
The immunologic release of constituents from neutrophil leukocytes. The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles.
J Immunol 107 1536-1546.

Henson PM and Johnson RB (1987).
Tissue injury in inflammation: oxidants, proteinases and cationic proteins.
J Clin Invest 79 669-674.

Henson PM (1976).
Membrane receptors on neutrophils.
Immunol Comm 5 757-774.

Henson PM and Oades ZG (1975).
Stimulation of human neutrophils by soluble and insoluble immunoglobulin aggregates.
J Clin Invest 56 1054-1061.

Hirasawa K and Nishizuka Y (1985).
Phosphatidylinositol turnover in receptor mechanism and signal transduction.
Ann Rev Pharm Toxicol 25 145-170.

Hirata F (1987).
The regulation of lipomodulin, a phospholipase inhibitory protein, in rabbit neutrophils by phosphorylation.
J Biol Chem 256 7730-7733.

Hirata F, Schiffmann E, Venicatasubramanian K, Solomon D and Axelrod J (1980).
A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids.
Proc Natl Acad Sci USA 77 2533-2536.

Hirata F, Matsuda K, Notsu Y, Haltori T and Delcarmine R (1984).
Phosphorylation at a tysroine residue of lipomodulin in thymocytes.
Proc Natl Acad Sci USA 81 4717-4721.

Hirsch JG and Cohn ZA (1960).
Degranulation of polymorphonuclear leukocytes following phagocytosis of micro-organisms.
J Exp Med 112 1005-1014.

Hochstein P and Jain SK (1981).

Association of lipid peroxidation and polymerization of membrane proteins with erythrocyte aging.
Fed Proc 40 182-188.

Horrobin DF, Jenbinok and Manku MS (1983).
Raynauds phenomenon, histamine and prostaglandins.
Lancet 747-8.

Huang KS, Wallner BP, Mattaliano RJ, Tuzzard R, Burre C, Frey A et al.
Two human 35 kd inhibitors of phospholipase A_2 are related to pp 60 and to the epidermial growth factor receptor/kinase.
Cell 46 191-199.

Hugli TE and Muller Eberhaard HJ (1978).
Anaphylatoxins, C3a and C5a.
Adv Immunol 26 1-53.

Hulstrom D and Sveryo R (1979).
Intravital and electron microscopic study of bradykinin induced vascular permeability changes using FITC dextran as a tracer.
J Pathol 129 125-136.

Hurd ER, Jasin HE, Gillam JN (1980).
Correlation of disease activity and Clq binding immune complexes with the neutrophil inclusions which form in the presence of SLE sera.
Clin Exp Immunol 40 383-391.

Hurst NP (1987).

The Molecular basis of activation and regulation of the phagocyte respiratory burst.
Ann Rheum Dis 46 465-272.

Hurst NP (1987).
Molecular basis of activation and regulation of the phagocyte respiratory burst.
Ann Rheum Dis 46 265-272.

Hystad RA (1981).
The protective action of ceruloplasmin on Fe^{2+} stimulated lysis of rat erythrocytes. Int J Biochem 13 221-224.

Ignarro LT, Lint TF and George WJ (1974).
Hormonal control of lysosomal enzyme release from human neutrophils.
J Exp Med 134 1395-1414.

Ignarro C and Cech SY (1976).
Bidirectional regulation of lysosomal enzyme secretion and phagocytosis in human neutrophils by guanosine 3^1 5^1 monophosphate and adenosine 3^1 5^1 monophosphate.
Proc Soc Exp Biol Med 151 448-452.

Ignarro LT and Metcalf JA (1977).
H_2O_2 release from human granulocytes during phagocytosis.
J Clin Invest 60 1266-12279.

Inoue M, Kishimoto A, Takai and Nishizuka I (1977).

Studies on a cyclic nucleotide independent protein kinase and its pro-enzyme in mammilian tissues.
J Biol Chem 252 7610-7616.

Iwanmoto H, Kobayashi T, Hasegawa E and Morita Y (1987).
Reaction of human myeloperoxidase with hydrogen peroxide and its true catalase activity.
J Biochem 101 1407-1412.

Iyer GYN, Islam MF and Quastel JH (1961).
Biochemical aspects of phagocytosis.
Nature 192 535-541.

Iyer GYN and Quastel JH (1963).
NADPH and NADH oxidation by guinea pig polymorphonuclear leukocytes.
Can J Biochem 41 427-434.

Jasin HE (1983).
Generation of IgG aggregates by the myeloperoxidase-hydrogen peroxide system.
J Immunol 130 1918-1923.

Jimenez SA (1983).
Cellular immune dysfunction and the pathogenesis of scleroderma.
Sem Arthritis Rheum 13 104-113.

Joke W, Horman-Muller T, Weening RS and Roos D (19875).

Production of hydrogen peroxide by phagocytosing human granulocytes.
J Lab Clin Med 85 198-207.

Jones HP, Ghai G, Petrone WF and McCord J (1982).
Calmodulin dependent stimulation of the NADPH oxidase of human neutrophils.
Biochim Biophys Acta 714 152-156.

Johnson RJ, Couser WG, Chi EY, Adler S and Klebanoff SJ (1987).
New mechanism for glomeruilar injury - myeloperoxidase-hydrogen peroxide-halide system.
J Clin Invest 79 1379-1387.

Just G (1907).
Kinetische intersuchung der autoxydation des in waser gelosten ferrobic arbonats.
Chem Ber 40 3695-4000.

Kahaleh MB, Shere GK and LeRoy EC (1979).
Endothelial injury in scleroderma.
J Exp Med 149 1326-1335.

Kahaleh MB, Osbom I and LeRoy EC (1982).
Elevated levels of circulating platelet aggregates and B thromboglobulin in scleroderma.
Ann Intern Med 96 610-613.

Kahaleh MB, Osbom I, LeRoy EC (1981).

Increased factor VIII/von Willebrand factor antigen and von Willebrand factor activity in scleroderma and Raynauds phenomenon.

Kahaleh MB, Scharstein K and LeRoy EC (1985).
Enhanced platelet adhesion to collagen in scleroderma. Effect of scleroderma plasma and scleroderma platelets.
J Rheumatol 12 468-471.

Kameda K, Ono T and Imai Y (1979).
Participation of superoxide hydrogen peroxide and hydroxyl radicals in NADPH cytochrome P450 reductase catalysed peroxidation of methyl linoleate.
Biochim Biophys Acta 572 77-

Kato H, Ishitoya J and Takewnawa T (1986).
Inhibition of insitol phospholipid metabolism and calcium mobilisation by cyclic-AMP increasing agents and phorbol esters in neutrophils.
Biochem Biophys Res Comm 139 1272-1278.

Kay A, Glass BJ and Salter D Mc G (1979).
Leucoattractants enhance complement receptors on human phagocytic cells.
Clin Exp Immunol 38 294-299.

Kelly KL and Wong EHA (1987).
Hormonal regulation of phospholipid methyltransferase by cyclic 3^1 5^1 adenosine monophosphate dependent and independent mechanisms.
Endocrinology 120 2421-2427.

Kikkawa U, Takai Y, Tanaka Y, Miyaka R and Nishizuja J (1983).
Protein kinase C as a possible receptor protein of tumour promoting phorbol esters.
J Biol Chem 258 11442-11445.

Kirby JDT, Dowd PM, Lima DRA, Kilfeather S and Turner P (1980).
Prostacyclin increases cyclic nucleotide responsiveness of lymphocytes from patients eith systemic sclerosis.
Lancet (ii) 453-4.

Kishimoto A, Takai Y, Mon T, Kikkaiva U and Nishuzuka Y (1980).
Activation of calcium and phospholipid dependent protein kinase by diacylglycerol. Its possible relation to phosphatidylinositol turnover.
J Biol Chem 255 2273-

Klebanoff SJ and Hamon CB (1972).
Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes.
J Reticuloendothel Soc 12 170-196.

Klebanoff SJ (1982).
The iron H_2O_2 cytotoxic system.
J Exp Med 156 1262-1267.

Klebanof SJ (1982).
Oxygen dependent cytotoxic mechamisms of phagocytes.
Adv Host Def Mech 1 111-162.

Koffer D, Schur PH and Kunkel HG (1967).
Immunological studies concerning the nephritis of sytemic lupus erythematosus.
J Exp Med 126 607-623.

Korchak HM, Vienne K, Rutherford LE, Wilkenfield, Finkelstein MC and Weissmann G (1984).
Stimulus response coupling in the human neutrophil: Temporal analysis of changes in cytosolic calcium and calcium efflux.
J Biol Chem 259 4076-4082.

Kovacs IB, Meynck Thomas PH, Mackay AR, Rustin MHA and Kirby JDT (1986).
Increased chemluminescence of polymorphonuclear leukocytes from patients with progressive systemic sclerosis.
Clinical Sci 70 257-261.

Kurata N, Hara I, Kinashi M, Akita H and Ofiji T (1978).
The correlaton of antibodies to nuclear ribonuclearprotein (RNP) and nucleic acid protein (Sm) with nephritis in patients with systemic lupus erythematosus.
Contrib Nephrol 9 17-24.

Kurimura Y, OIchiai R, Matsuura N (1968).
Oxygen oxidation of ferrous ions induced by chelation.
Bull Chem Soc Jap 41 2234-2239.

Lakha S (1978).

Immune complexes.
Clin Rheum Dis 9 199-225.

Lamb AB, Elder LW (1931).
The electromotive activation of oxygen.
J Am Chem Soc 53 137-163.

Lambert PH and Casali P (1978).
Immune complexes and the rheumatic diseases.
Clin Rheum Dis 4 617-642.

Lee P, Lonolly N, Sukenik S and Alderdice A (1985).
The clinical significance of coagulation abnormalities in systemic sclerosis (scleroderma).
J Rheumatol 12 514-517.

Lefkowitz RJ (1979).
Ann Intern Med 91 450-

Lefkowitz RJ and Hoffman BB (1981).
New directions in adrenergic receptor research.
in Towards Understanding Receptos pp 61-70.
ed Lanble JW, Elsevier, North Holland.

Lentek AL, Schreiber AD and MacGregor (1976).
The induction of augremented granulocyte adherence by inflammation mediation of a plasma factor.

J Clin Invest 57 1098-1103.

LeRoy EC (1972).
Connective tissue synthesis by scleroderma skin fibroblasts in cell culture.
J Exp Med 135 1351-1362.

Lewinsohn DM, Bargatze RF and Butcher EC (1987).
Leukocyte-endothelial cell recognition: evidence of a common molecular mechanism shared by neutrophils, lymphocytes and other leukocytes.
J Immunol 138 4313-4321.

Lin PS, Kwock L and Goodchild NT (1980).
Copper chelator enhancement of bleomycin cytotoxicity.
Cancer 46 2360-2364.

Litosch I (1987).
Regulatory GTP-binding proteins; emerging concepts on their role in cell function.
Life Sci 41 251-258.

Little C and O'brien PJH (1968)
The effectiveness of a lipid peroxide in oxidising protein and non protein thiols.
Biochem J 106 419-423.

Lloyd W and Schur PH (1981).

Immune complexes, complement and anti-DNA in exacerbations of systemic lupus erythematosus.
Medicine 60 208-217.

Lockcroft S, Barrowman MM and Comperts (1985).
Breakdown and synthesis of polyphosphoinositides in f-met-leu-phe stimulated neutrophils.
FEBS letts 181 259-263.

Lockroft S and Comperts BD (1985).
Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase.
Nature 314 534-536.

Lucy JA (1972).
Functional and structural aspects of biological membranes. A suggested role of vitamin E in the control of membrane permeability and stability.
Ann N Y Acad Sci 203 4-11.

Lunec J (1984).
Free radical-mediated aggregation of human IgG stimulates neutrophils to generate superoxide radicals.
Agents Actions 15 37-38.

Majno G and Palade GE (1961).
Studies on inflammation I. The effect of histamine and serotonin on vascular permeability. An electron microscopic study.

J Biophys Biochem Cytol 11 571-597.

Majno G, Shea SM and Heventhal M (1969).
Endothelial contraction induced by histamine type mediators. An electron microscopic study.
J Cell Biol 42 647-672.

Malech HL, Root RK and Gallin JL (1977).
Structural analysis of human neutrophil migration: microtubule and microfilament orientation and function during chemotaxis.
J Cell Biol 75 669-693.

Manganiello V, Evans IJH, Stossel TY, Mason PT and Vaughan M (1971).
The effect of polystyrene beads on cyclic 3^1 5^1 adenosine monophosphate concentration in leukocytes.
J Clin Invest 50 2741-1744.

Mantovani B (1975).
Different roles of IgG and complement receptors in phagocytosis by polymorphonuclear leukocytes 115 15-

Maridonneau-Parini I and Tauber AI (1986).
Activation of NADPH oxidase by arachidonic acid invovles phospholipase A_2 in intact human neutrophils but not in the cell free system.
Biochem Biophys Res Commun 138 1099-1106.

Maridonneau-Parini I, Tringale SM and Tauber TI (1986).

Identification of distinct activation pathways of the human neutrophil NADPH-oxidase.
J Immunol 137 2925-2931.

Maslen CL (1986).
The effects of anti-inflammatory compounds on the oxidative metabolisms of human phagocytic cells.
PhD Thesis University of Bath.

Maslen CL, Stevens TRJ and Hall ND (1987.
The generation of lipid peroxides by stimulated human nuetrophils.
J Immunol Met 98 71-76.
PhD thesis Univeristy of Bath.

McArthur W, Derr K and Dixon M (1982).
Immune modulation of connective tissue fucntions: Studies on the production of a collagen synthesis inhibitory factor by a population of human peripheral blood monoculcear cells.
Cell immunol 76 126-139.

McCarty CA, Rice JR, Bembe ML and Barada FA (1983).
Clinical correlations and association with favorable prognosis in patients with scleroderma variants.
Arthritis Rheum 26 1-7.

McCluskey RT, Hall CL and Colvin RB (1981).
Immune complex mediated diseases.
Human Pathol 9 71-84.

McCord JM and Fridovich [2] (1969).
Superoxide dismutase: An enzyme function for erythocuprein (hemocuprein).
J Biol Chem 244 6049-6055.

McCord JM, Wong K, Stokes SH, Petrone NF and English D (1980).
Superoxide and inflammation: a mechanism for the anti-inflammatory activity of superoxide dismutase.
Acta Physiol Scand 492 25-30.

McCord JM (1980).
Oxygen free radicals: release and activities. A superoxide activated chemotactic factor and its role in the inflammatory process,
Agents Actions 10 522-527.

McCord JM (1985).
Oxygen derived free radicals in post-ischaemic tissue injury.
N Eng Med 312 159-163.

McCutcheon M (1946).
Chemotaxis in leukocytes.
Physiol Rev 26 319-337.

McIntyre T, Zimmerman GA and Prescott SM (1986).

Leukotriene C_4/D_4 stimulate human endothelial cells to synthesize platelet activating factor and bind neutrophils.
Proc Natl Acad Sci (USA) 83 2004-2208.

McRipley RJ and Sbarra AJ (1967).
The role of the phagocyte in host paracite interactions XIII Hydrogen peroxide - myeloperoxidase bactericidal system in the phagocyte.
J Bacteriol 94 1425-1430.

Mead JF (1976).
Free radical mechanisms of lipid damage and consequneces for cellular membranes, in Free Radical in Biology pp 51-62.
ed Pryor WA Academic Press, New York.

Mead JF (1976).
Free radical mechanisms of lipid damage and consequences for cellular membranes in Free Radicals in Biology.
ed Pryor WA, pp 51-68, Academic Press, New York.

Meister A and Anderson MC (1983).
Glutathione.
Ann Rev Biochem 52 711-760.

Mello AC, and Meneghini R (1984).
In vivo formation of single strand breaks in DNA by hydrogen peroxide is mediated by the Haber Weiss reaction.
Biochim Biophys Acta 781 56-63.

Melloni E, Pontremoli S, Michetti M, Sacco O, Sparatone B and Horecker BL (1986).
The involvement of calpain in the activation of protein kinase C in neutrophils stimulated by phorbol myristate acetate.
J Biol Chem 261 4101-4105.

Miesher PA, Paronetto F and Lambert PH (1979).
Systemic lupus erythematosus.
In Textbook of Immunopathology pp 963-1001.
ed Miescher PA and Muller-Eberhard HJ, Grine and Stratton, New York.

Moore L, Chen T, Knapp HR and Landon EJ (1975).
Energy dependent calcium sequestration activity in rat liver microsomes.
J Biol Chem 250 4562-4568.

Motohashi N and Mon I (1983). Superoxide dependent formation of hydroxyl radical catalysed by transferrin.
FEBS 157 197-199.

Mozeley PL, Hemkem C and Hunninghake GW (1986).
Augmentation of fibroblast proliferation by bleomycin.
J Clin Invest 78 1150-1154.

Naccache PH, Molski P, Borgeat P and Shaasfi RI (1981).
Mechanism of action of LTB_4. Intracellular calcium redistribution in rabbit neutrophils.
J Cell Physiol 118 13-18.

Naqui A and Chance B (1986).
Reactive oxygen intermediates in biochemistry.
Ann Rev Biochem 55 137-166.

Neer EJ, Lok JM and Wolf LG (1984).
Purification and properities of the inhibitory guanine nucleotide regulatory unit of brain adenylate cylclase.
J Biol Chem 259 14222-14229.

Negoso N, Kanayama Y, Takeda T, Amatsu K, Koda S, Inoue Y, Kim T, Okamura M and Inoue T (1987).
Clinical significance of U_1 RNP immune complexes in mixed connective tissue disease and systemic lupus erythematosus.
Rheumatol Int 7 7-11.

Niedel JE, Kuhn LJ and Vandenbark GR (1985).
Phorbol diester copurifies with protein inase C.
Proc Natl Acad Sci USA 80 36-40.

Nishizuka Y (1984).
The role of protein kinase C in cell surface signal transduction and tumour promotion.
Nature (Lond) 308 693-698.

Northup JK, Smigel MD, Stenweis and Gilamn AG (1983).
The subunits of the stimulatory regulatory component of adenylate cyclase.

J Biol Chem 258 11469-11476.

Ohlsson K (1978).
Interaction of granulocyte neutral proteases with $_1$ antitypsin, $_2$ macroglobulin and $_1$ antichymotrypsin.
In Neutral proteases of human polymorphonuclear pp 167-177.
Leukocytes.
eds Hasemann K and Janoff D, Urban and Schwazenberg, Baltimore.

Ohno Y and Gallin JI (1985).
Diffusion of extracellular H_2O_2 into intracellular compartments of human neutrophils.
J Biol Chem 260 8443-8446.

Okamura N, Ohasi S, Nagahisa N and Ishibashi S (1984).
Changes in protein phosphorylation in guinea pig polymorponuclear leukocytes by treatment with membrane-perturbing agents which stimulate superoxide anion production.
Arch Biochem Biophys 228 270-277.

Oshtizuka T, Ozawa M, Okamoto T, Uxhida M, Okamura N and Ishibashi S (1987).
Significance of phosphorylation/dephosphorylation of 46K protein (S) in regulation of superoxide anion production in intact guinea pig polymorphonuclear leukocytes.
J Biochem 101 897-903.

Oseas R, Yang H, Baehner RL and Boxer LA (981).
Lactoferrin. A promoter of polymorphonuclear leukocyte adhesiveness.
Blood 57 939-943.

Oseas R, Allen J, Yang H, Baehner RL and Boxer LA (1982).
The mechanism of dexamethasone inhibition of chemotactic factor induced granule release.
Blood 59 265-271.

Orrenius S (1985).
Biochemical mechanisms of cytotoxicity.
TIPS (FEST suppl) 15-20.

Packer JE, Slater TF and Wilson R (1979).
Direct observation free radical interactions between vitamin E and vitamin C.
Nature (Lond) 278 737-738.

Palmbad J (1984).
The role of granulocytes in inflammation.
Scand J Rheumatol 13 163-172.

Panosian JO and Marinetti GV (1983).
α_2 adrenergic receptors in human polymorphonulcear leucocyte membranes.
Biochem Pharmacol 32 2243-2247.

Pascoe GA, Fariss MW, Olafdottir K and Reed DJ (1987).

A role of vitamin E in protection against cell injury: Maintenance of intracellular glutathione.
Eur J Biochem 166 241-247.

Pearson JD, Carlton JS, Beesley JE, Hutchings A and Gordon JL (1979).
Granulocyte adhesion to endothelium in culture.
J Cell Science 38 225-235.

Pepinsky RB and Sinclair LK (1986).
Epidermal growth factor dependent phosphorylation of lipocortin.
Nature 321 81-84.

Peters JH, Ginsberg, Bohl BP, Skiar LA and Cochrane G (1986).
Intravascular release of intact cellular fibronectin during oxidant induced injury of the in vitro perfused rabbit lung.
J Clin Invest 78 1596-1603.

Petrone WE, English DK, Wong K, McCord JM (1980).
Free radicals and inflammation: Superoxide dependent formation of a neutrophil chemotactic factor.
Proc Natl Acad Sci USA 77 1159-1163.

Pham-Hull T, Chollet-Martin S, Pevianin A, Marquetty C, Sourbier P, Babin-Chevaye C, Olive D, Gougerot-Pocidalo MA, Debre P and Hakim J (1987).
Comparison of the blocking effects of monoclonal antibodies MO1-d and LFA-1 X in human neutrophils.
Immunol 62 61-67.

Pick E and Keisari (1980).
A simple colourimetric method for the measurement of hydrogen peroxide produced by cells in culture.
J Immunol Meth 38 161-170.

Pick E and Muzel D (1981).
Rapid micro assays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automated enzyme immunoassay reader.
J Immunol Meth 46 211-227.

Robinson DR (1985).
Low molecular weight mediators of inflammation: prostaglandins, leukotreines and cyclic nucleotides.
In The textbook of Rheumatology pp 71-83.
Ed Kelley WN, Harris ED, Ruddy S and Sledge CB.
Publishers Saunders, London.

Rosen H and Klebanoff SJ (1979).
Bactericidal activity of a superoxide anion generating system: A model for the polymorphonuclear leukocyte.
J Exp Med 149 27-39.

Posner AM (1953).
The kinetic of auto oxidation of ferrous ions in concentrated HCL solutions.
Trans Faraday Soc 49 382-388.

Potremou S, Melloni E, Salamuno Fm Sparatose B, Michetti M, Sacco O and Horecker BL (1986).
Phosphorylation of proteins in human neutrophils activated with phorbol mynstate acetate or with chemotactic factor.
Arch Biochem Biophys 250 23-29.

Queensbury PJ and Gibone MA (1980).
Vascular endothelium as a regulator of granulopoiesis.
Blood 56 1060-1067.

Rink TJ, Sanelez A and Hallam TJ (1983).
Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets.
Nature 305 317-319.

Riboldi P, Asero R, Origgi L, Crespi S, Meroni PL, Sguotti C and Sabbadini MG (1985).
Antinuclear antibodies in progressive systemic sclerosis.
Clin Exp Rheum 3 205-211.

Roberts MF, Deems RA, Mincey TC and Dennis EA (1976).
Chemical modification of the histidine residue in phospholipase A_2 (Naja naja naja).
J Biol Chem 282 2405-2411.

Rodbell M (1980).

The role of hormone receptors and GTP regulatory proteins in membrane transduction.
Nature 284 17-22.

Rodnan GP, Myerowitz RL and Justh GO (1980).
Morphological changes in the digital arteries of patients with progressive systemic sclerosis (scleroderma) and Raynauds phenomenon.
Medicine 59 398-408.

Rodnem H (1979).
Progressive systemic sclerosis (scleroderma).
In Arthritis and Allied conditions pp 762-809.
Ed Hollander JL and McCarthy DL.
Publishers Lea and Febiger, Philadelphia.

Roos D, Weening RS and Wyss RR (1980).
Protection of human neutrophils by endogenous catalase: Studies with cells from catalase deficient individuals.
J Clin Invest 65 1515-1522.

Root RK and Metcalf JA (1977).
H_2O_2 release from human granulocytes during phagocytosis.
J Clin Invest 60 1266-1279.

Ruggieri A and LeRoy EC (1986).
Scleroderma (systemic sclerosis).
In Rheumatology and Immunology pp 257-268.

Ed Cohen AS and Bennet JC.

Grune and Stratton Orlando, USA.

Ryan TJ (1980).

Anatomical features relevant to vasculitis.

In Vasculitis pp 25-30.

Ed Wolf K and Winkelmann PK, Lloyd-Duke, London.

Sakata A, Ida E, Tominga M and Onoue K (1987).

Arachidonic acid acts as an intracellular activator of NADPH oxidase in Fc receptor mediated superoxide generation in macrophages.

J Immunol 138 4353-4359.

Salin ML and McCord JM (1974).

Superoxide dismutase in polymorphonuclear leukocytes.

J Clin Invest 54 1005-1009.

Salmon SE, Cline MJ, Schultz J (1970).

Myeolperoxidase deficiency: immunological study of a genetic leukocyte defecit.

N Eng J Med 282 250-253.

Sbarra AJ and Kamovsky ML (1959).

The biochemical basis of phagocytosis metabolic changes during the ingestion of particles by polymorphonulcear leukocytes.

J Biol Chem 234 1355-1362.

Scott WA, Pawlowski NA, Murray HW, Andreach M, Zrike J and Cophn Z (1982).
Regulation of arachidonic acid metabolism by macrophage activation.
J Exp Med 155 1148-1153.

Scherer NM and Deamer DN (1986).
Oxidative stress impairs the function of the sacroplasmic reticulum by oxidation of sulphydryl groups in the Ca^{2+} ATPase.
Arch Biochem Biophys 246 589-601.

Schiffmann E, Corcoran BA and Cuahl SM (1975).
N formyl methionyl peptides as chemoattractant for leukocytes.
Proc Nat acad Sci USA 72 1059-

Schneider C, Zanetti M and Romeo D (1981).
Surface reactive stimuli selectively increase protein phosphorylation in human neutrophils.
FEBS Lett 127 4-8.

Schur PH (1986).
Systemic lupus erythematosus.
In Rheumatology and Immunology pp 247-257.
Ed Cohen AS and Bennett JC, Grune and Stratton, London.

Schrud-Schonbein GN (1987).
Capillary plugging by granulocytes and the no reflow phenomenon in the microcirculation.

Fed Proc 46 2397-2401.

Schwabe U (1982).
Direct binding studies of adenosine receptors.
In More about Receptors pp 140-147.
Ed Lambe JN, Elsevier Holland.

Segal AW, Heyworth PG, Cockrofts and Barrowman MM (1985).
Stimulated neutrophils from patients with autosomal recessive chronic granulmatous disease fail to phosphorylate a Mr-44,000 protein. Nature 316 547-558.

Segal AW, Jones OTG, Webster D, Allison AC (1978).
Absence of a newly described cytochrome b from neutrophils of patients with chronic granulomatosus disease.
The Lancet (8) 446-449.

Segal AN, Dorling J and Coade S (1980).
Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes.
J Cell 85 42-59.

Segal AW and Jones OTG (1978).
Novel cytochrome b system in phagocytic vacuoles of human granulocytes.
Nature 276 515-517.

Shaafi RI and Molski TFP (1987).

Signalling for increased cytoskeletal actin in neutrophils.
Biochem Biophys Res Com 145 934-941.

Sharkey NA, Leach KL and Blumberg PM (1984).
Competitive inhibition by diacylglycerol of specific phorbol ester binding.
Proc Natl Acad Sci USA 81 607-610.

Sharp GC, Irwin WS, Tan EM, Gould RG and Holman HR (1972).
Mixed connective tissue disease - an apparently distinct rheumatic disease syndrome associated with a specific antibody to an extractable nuclear antigen.
Am J Med 52 148-159.

Shields JM and Haston WS (1985).
Behaviour of neutrophil leucocytes in uniform concentrations of chemotactic factors: contraction waves, cell polarity and persistence. J Cell Sci 74 75-93.

Sies H and Akerboom TPM (1984).
Guitathione disulphide efflux from cells and tissues.
Methods enzymol 105 445-451.

Smith MJH (1981).
Leukotriene B_4.
Gen Pharmacol 12 211-216.

Simchowitz L, Atkinson JP and Spilberg I (1982).

Stimulation of the respiratory burst in human neutrophils by crystal phagocytosis.
Arth Rheum 25 181-188.

Simchowitz L and Spilberg I (1979).
Generation of superoxide radicals by human peripheral neutrophils activated by chemotactic factor. Evidence for the role of calcium.
J Lab Clin Med 93 583-586.

Sinclair RA, Antonovych TT and Mostofi FK (1976).
Renal proliferative arteriopathies and associated glomenular changes. A light and electonmicroscopic study.
Human Pathol 7 565-577.

Smedly LA, Tonnersen MG, Sandhaus RA, Haslett C, Gutherie LA, Johnson RB, Henson PM and Worthen GS (1986).
Neutrophil mediated injury to endothelial cells. Enhanced by endotoxin and the essential role of neutrophil elastase.
J Clin Invest 77 1233-1244.

Smolen JE, Korchak HM and Weissmann G (1981).
The roles of extracellular and intracelular calcium in lysosomal enzyme release and superoxide anion generation by human neutrophils.
Biochim Biophys Acta 677 512-520.

Smolen JE and Weissmann G (1980).

Effects of indomethacin, 5, 8, 11, 14 eicosatryenoic acid and p bromophenacyl bromide on lysosomal enzyme release and superoxide generation by human polymorphonuclear leukocytes.
Biochem Pharmacol 29 533-538.

Smolen JE, Korchak HM and Weissmann G (1980).
Increased levels of cyclic adenosine 3 5 monophosphate in human polymorphonuclear leukocytes after surface stimulation.
J Clin Invest 65 1077-1083.

Streb H, Irvine RF, Berridge NJ and Schulz I (1983).
Release of Ca^{2+} from non mitochondrial intracellular store in pancreatic acinar cells by inositol 145 triphosphate.
Nature 306 67-69.

Stossel TP (1976).
Phagocytosis.
New Eng J Med 290 717-723.

Stossel TP (1973).
Quantitative studies of phagocytsis: kinetic effects of cations and heat lable opsonins.
J Cell Biol 58 346-356.

Subak ML and Sharpe JH (1981).
Int Rev Cytol 69 45-104.

Sulsef (1987).
New perspectives on the mode of action of antidepressant drugs.
In Towards understanding receptors pp 990104.
ed Lamble JW, Elsevier, North Holland.

Sausville EA, Peisach T and Horwitz SB (1978).
Biochemistry 77 1150-

Sutton HC (1985).
Efficiency of chelated iron compounds as catalysts for the Haber-Weiss reaction.
J Free Rad Biol Med 1 195-202.

Suzuki Y and Hehrer RI (1980).
NADPH oxidase activity stimulated by phorbol myristate acetate.
J Clin Invest 66 1409-1418.

Takenawa T, Ishitoya J, Homma Y, Kato M and Magai Y (1985).
Role of enhanced inositol phospholipid metabolism in neutrophil activation.
Biochem Pharmacol 34 1831-1935.

Tan EM, Rodnan GP, Garcia I, Moroi Y, Fritzler MJ and Peebles C (1980).
Diversity of antinuclear antibodies in progressive systemic sclerosis. Anticentromere antibody and its relationship to Crest syndrome.

Arthritis Rheum 23 617-25.

Tappel AL and Dillard CJ (1981).
In vivo lipid peroxidation: Measurement via exhaled pentane and protection by vitamin E.
Fed Proc 40 174-178.

Taylor DL and Condeelis JS (1979).
PMN chemotaxis in vitro, a consequence of Na^+/H^+ inhibition is an alteration in intracellular pH.
Int Rev Cytol 56 57-144.

Tauber A (1987).
Protein kinase C and the activation of the human neutrophil NADPH oxidase.
Blood 69 911-720.

Thor H, Smith MT, Hartizell P, Bellomo G, Jewell SA and Orrenius S (1982).
The metabolism of menadione by isolated hepatocytes: a study of the implications of oxidative stress on intact cells.
J Biol Chem 257 12419-12425.

Turner R, Mashburn R, Collins L, De Chatelet L and Kaufmann T (1977).
Rheumatoid factor - immunoglbulin G complex precipitation and neutrophil stimulation as an in vtiro model for rheumatoid inflammation.
In Perspectives in Inflammation p 39.
Ed Willoughby DA. MTP press Lancaster.

Vollet JJ, Roth LE and Davidson M (1972).
Cell surface activity associated with fusion and retraction in protazoan cells.
J Cell Biol 55 269a.

Verghese MW, Fox K, McPhail LC and Snyderman R (1985).
Chemoattractant elicited alteration of cAMP levels in human polymorphonuclear leukocytes requires a calcium dependent mechanism which is independent of transmembrane activation of adeylate cyclase.
J Biol Chem 260 6760-

Via CS and Oades ZG (1975).
Stimulation of human neutrophils by soluble and insoluble immunoglobulin aggregates.
J Clin Invest 56 1053-1061.

Vissers MCM and Winterbourn CC (1987).
Myeloperoxidase-dependent oxidative inactivation of neutrophil neutral proteinases and microbicidal enzymes.
Biochem J 245 277-280.

Walling C (1982).
The Nature of the primary oxidants in oxidations mediated by metal ions.
In Oxidases and related redox systems.
eds King TE, Mason HS, Morrisson M, pp 85-97, Pergamon Press, New York.

Walsh DA, Perkins JP and Krebs EG.

J Biol Chem 243 3763.

Walsh CE, Waite BM, Thomas MJ and De Chatelet LR (1981).
Release and metabolism of arachidonic acid in human neutrophils.
J Biol Chem 256 7228-7234.

Walsh CE, De Chatelet LR, Chilton FH, Wykle RL and Waite M (1983).
Mechanisms of arachidonic acid release in human neutrophils.
Biochim Biophys Acta 750 32-40.

Wedmore CV and Williams TJ (1981).
Control of vascular permeability by polymorphonuclear leukocytes in inflammation.
Nature (London) 289 646-650.

Weiss SJ and Ward PA (1982).
Immune complex induced generation of oxygen metabolites by human neutrophils.
J Immunol 129 309-313.

Weiss SJ and LoBuglio AF (1982).
Biology of disease: Phagocyte generated oxidants and cellular injury.
Lab Invest 47 5-18.

Weissman G (1979).
Mediators of tissue damage in rheumatoid arthritis: phagocytes as secretory organs of rheumatoid inflammation.
Triangle Sandoz J Med Sci 18 45-52.

Weissman G (1987).
Pathogenesis of inflammation. Effects of the phamacological manipulation of arachidonic acid metaboism on the cytological response to inflammatory stimuli.
Drugs 33 (suppl 1) 28-37.

Weissman G (1983).
Pathways of arachidonate oxidation to prostaglandins and leukotrienes.
Sem Arth Rheum 13 123-129.

Werb Z and Cohn ZA (1972).
Plasma membrane synthesis in the macrophage following phagocytsis of polystyrene latex particles.
J Biol Chem 247 2439-2446.

White JR, Naccache PH, Molski THP, Borgeat P and Shaafi RI (1983).
Direct demonstration of increased intracellular concentration of free calcium in rabbit and human neutrophils following stimulation by chemotactic factor.
Biochem Biophys Res Comm 113 44-50.

Wickens DG and Dormandy TL (1984).
Effect of free-radical activity on human gamma globulin.
Agents Actions 15 47-48.

Williams TJ (1985).
Vascular responses and their suppression: Vasodilation and Oedema in the Pharmacology of Inflammation pp 49-60.
Ed Bonta IL, Bray MA, Parnham MJ, Elserier, Oxford.

Williams TJ and Morley J (1973).
Prostaglandins as potentiators of increased vascular permeability in inflammation.
Nature (Lond) 246 215-217.

Williams TJ and Jose PJ (1981).
Mediators of increased vascular permeability after complement activation: histamine independent action of rabbit C_{5a}.
J Exp Med 153 136-142.

Winterbourn CC (1979).
Comparison of superoxide with other reducing agents in the biological production of hydroxyl radicals.
Biochem J 182 625-628.

Winterbourn CC, Garua R and Segal AW (1985).
Production of the superoxide adduct of myeloperoxidase (compound 111) by stimulated neutrophils, and its reactivity with H_2O_2 and chloride.
Biochem J 228 583-592.

Winterbourn C (1986).
Myeloperoxidase as an effective inhibitor of hydroxyl radical producyion.
J Clin Invest 78 545-550.

Wolff SP, Garner A and Dean RT (1986).
Free radicals, lipid and protein degradation.
TIBS 27-32.

Wolfson M, McPhail LC, Nasrallah VN and Syndernam R (1985).
Phorbol myristrate acetate mediates redistribution of protein kinase C in human neutrophils: Poteintial role in the activation of the respiratory burst enzyme.
J Immunol 135 2057-2062.

Wolfson M, McPhail LC, Nasrallah VN and Synderman R (1985).
Phorbol mynstate acetate mediates redistribution of protein kinase C in human neutrophils: Potential role in the activation of the respiratory burst enzyme.
J Immunol 135 2057-2064.

Wright AE and Douglas SR (1903).
An experimental investigation of the role of body fluids in connection with phagocytsis.
Proc Biol Soc (Lond) 72 357-366.

Wright DG and Gallin JI (1979).
Secretory responses of specific granules by human neutrophils during adherence in vitro and during exudation in vivo.
J Immunol 123 285-292.

Wright DG (1981).
The neutrophil as a secretory organ of host defence.
In Infection - The physiologic and metabolic response of the host pp1-30.
Ed Powanda MC and Ganonico RG, Elsevier, Holland/New York.

Yoshimasa T, Sibley DR, Bouvier M, Hefkowitz RJ and Caron MG (1987).
Cross talk between cellular signalling pathways suggested by phorbol ester induced adenylate cyclase phosphorylation.
Nature 327 67-70.

Zsanagy I and Floyd RA (1984).
Hydroxyl free radical reactions with amino acids and proteins studied by electron spin resonance spectroscopy and spin trapping.
Biochim Biophys Acta 790 238-250.